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Lipoic acid increases *de novo* synthesis of cellular glutathione by improving cystine utilization

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Abstract. Lipoic acid (thiotic acid) is being used as a dietary supplement, and as a therapeutic agent, and is reported to have beneficial effects in disorders associated with oxidative stress, but its mechanism of action remains unclear. We present evidence that lipoic acid induces a substantial increase in cellular reduced glutathione in cultured human Jurkat T cells, human erythrocytes, C6 glial cells, NB41A3 neuroblastoma cells, and peripheral blood lymphocytes. The effect depends on metabolic reduction of lipoic acid to dihydrolipoic acid. Dihydrolipoic acid is released into the culture medium where it reduces cystine. Cysteine thus formed is readily taken up by the neutral amino acid transport system and utilized for glutathione synthesis. By this mechanism lipoic acid enables cystine to bypass the x_c^- transport system, which is weakly expressed in lymphocytes and inhibited by glutamate. Thereby lipoic acid enables the key enzyme of glutathione synthesis, γ-glutamylcysteine synthetase, which is regulated by uptake-limited cysteine supply, to work at optimum conditions. Flow cytometric analysis of freshly prepared human peripheral blood lymphocytes, using monobromobimane labeling of cellular thiols, reveals that lipoic acid acts mainly to normalize a subpopulation of cells severely compromised in thiol status rather than to increase thiol content beyond physiological levels. Hence lipoic acid may have clinical relevance in restoration of severely glutathione deficient cells.

Keywords: ASC transporter, cysteine uptake, Jurkat cells, peripheral blood lymphocytes, thiols, x_c^- transporter

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1. Introduction

Modulation of cellular glutathione (GSH) status has long been discussed as a potential therapeutic strategy [1]. GSH depletion has been shown to sensitize cells to radiation and was also reported to overcome phenylalanine-mustard resistance of human ovarian tumor cells in experimental animals [2]. In contrast, elevation of cellular GSH may be expected to support GSH-dependent pathways, in particular hydroperoxide metabolism in diseases associated with oxidative stress, such as adult respiratory distress syndrome [3], idiopathic pulmonary fibrosis [4], Wilson's disease [5], Parkinson disease [6–8] and AIDS [9].

Dramatically decreased GSH levels may result from different phenomena: i) GSH is continuously released from most cells via the sinusoidal (liver) or canalicular (ubiquitous) GSH transporters [10] and rapidly cleared from the circulation mainly by renal degradation [11]. ii) Oxidized glutathione (GSSG), formed during enzymatic hydroperoxide removal [12] or non-enzymatic reactions, is exported from cells [13–16]. iii) Although the ubiquitous canalicular and the sinusoidal GSH transport systems work bidirectionally in principle, net GSH uptake is negligible due to the concentration gradient and the fast extracellular degradation [10], and uptake of intact GSH by the recently reported sodium-dependent GSH uptake system appears to be restricted to special tissues, e.g., brain endothelium [17]. Maintenance of cellular GSH therefore depends on its *de novo* synthesis in most tissues. iv) Intracellular cysteine is a limiting factor of GSH synthesis [1] and its availability through the neutral amino acid transport system ASC (for a review see Bannai [18]) is impaired in oxidative stress due to extracellular oxidation of cysteine to cystine. v) Uptake of cystine by the x_c^- transporter is inefficient in many cells and often compromised by competitive inhibition of glutamate using the same transporter [19]. Inadequate cystine utilization due to high serum glutamate levels has been demonstrated in HIV-infected patients [20], and may contribute to the loss of GSH in affected lymphocytes and impairment of function [21–23]. Similarly, the inhibition of cystine transport by glutamate has been implicated in glutamate-induced neurotoxicity [24].

Strategies to increase cellular GSH include: i) application of permeable labile esters of GSH; ii) providing cysteine derivatives as precursors of GSH synthesis which penetrate cells readily and are less easily oxidized than cysteine, such as N-acetyl-cysteine or *L*-2-oxothiazolidine-4-carboxylate; iii) treatment with antioxidants sparing GSH consumption (for a review see Meister [1]).

More recently, lipoic acid (LA) has been shown to substantially increase cellular GSH *in vitro* [25] and *in vivo* [26] and to prevent the loss of cellular thiols due to ischemia reperfusion [27]. Lipoic acid also inhibits the activation of the nuclear transcription factor (NF-κB) [28,29], may restore compromised lymphocyte functions of HIV-infected patients [30], and inhibits viral replication [31]. Similar effects have been reported for N-acetyl-cysteine treatment and attributed to the increase or restoration of cellular GSH pools [23,32–35]. This parallelity between LA and N-acetyl-cysteine is surprising, since LA itself can neither be rated as a GSH precursor, nor a GSH mimic, but rather consumes reduction equivalents during its metabolic conversion to dihydrolipoic acid (DHLA). LA quenches hydroxyl radicals, singlet oxygen and hypochlorous acid [36] but it shares this ability with a wide variety of endogenous compounds present abundantly in tissues. We therefore reasoned that the specific antioxidant action of LA may be mediated by the increase of cellular GSH and investigated the underlying mechanism systematically. Using human Jurkat lymphoma cells, in which LA effects [25, 37] and cysteine and cystine transport rates [38] have been well characterized, evidence is presented here that the increase of cellular GSH upon LA exposure is due to the metabolic reduction of LA to DHLA, release of DHLA into the medium, extracellular non-enzymatic reduction of cystine by DHLA, and uptake of cysteine by the ASC transporter, thus bypassing the glutamate-sensitive x_c^- transport system.

2. Methods

2.1. Materials

R, S, (racemic) LA, racemic tetranorlipoic acid and racemic DHLA were provided by ASTA Medica (Frankfurt, Germany). GSH, L-cystine, L-cysteine, L-serine, L-glutamic acid, octanoic acid, dehydroepiandrosterone (DHEA), oxidized D-L-dithiothreitol, dimethyl sulfoxide (DMSO), 2-mercaptoethanol (2ME), and D-L-buthionine-(S, R) sulfoximine (BSO), were purchased from Sigma Chemical Co. (St. Louis, MO). Monobromobimane was purchased from Molecular Probes (Eugene, OR). RPMI 1640, cystine free RPMI 1640 medium, Dulbecco's Modified Eagle's Medium and Dulbecco's Phosphate Buffered Saline (PBS) were obtained from Life Technologies (Gaithersburg, MD). Fetal bovine serum, sodium pyruvate, and penicillin-streptomycin were obtained from UCSF Cell Culture Facility (San Francisco, CA). HPLC grade solvents were purchased from Fisher scientific (Fair Lawn, NJ). Cystine [^{35}S] was obtained from DuPont NEN (Boston, MA).

In all the experiments, glutamate, 2-mercaptoethanol, tetranorlipoic acid, oxidized D-L-dithiothreitol, and D-L-buthionine-(S, R) sulfoximine were dissolved in phosphate buffered saline for treatment of cells. DHEA, DHLA, and lipoamide were solublized in dimethyl sulfoxide. Lipoic acid was solublized in PBS or, in comparative studies with DHLA and lipoamide, dissolved in DMSO. All the solutions were made sterile by filtration through a 0.2 μm Millipore (Bedford, MD) filter prior to use. Control samples were always treated with the corresponding amount of PBS or DMSO.

2.2. Cell culture conditions and treatment

Jurkat cells, clone E6-1 (human acute T cells leukemia), C6 glial cells (rat glial tumor cell line) and NB41A3 (mouse neuroblastoma cell line) were purchased from American Type Culture Collection (Bethesda, MD).

Jurkat cells were routinely cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in RPMI 1640 supplemented with 10% fetal bovine serum, sodium pyruvate (11 μg/ml), penicillin (100 units/ml), and streptomycin (100 units/ml). The day of the experiment, Jurkat cells were resuspended in fresh medium at a final concentration of $0.8 \times 10^6 - 0.9 \times 10^6$ cells/ml. Sterile aliquots of LA and other compounds were added to the culture medium.

Both C6 and NB41A3 cell were routinely grown in Dulbecco Modified Eagle's Medium supplemented with 10% serum and penicillin (100 units/ml), and streptomycin (100 units/ml) at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. In the late log phase of growth (2.5×10^6 cells/dish) the cells were supplied with fresh medium and treated with various compounds for up to 24 hours.

Cell numbers were determined with a hemocytometer. Cell viability was assayed using the trypan dye exclusion method. Lipoic acid and other compounds administered to the cells had no significant effect on cell viability.

2.3. Isolation and treatment of human peripheral blood lymphocytes and erythrocytes

Human blood was drawn from healthy male volunteers at the Tang Center Laboratory (Berkeley, CA). Peripheral blood lymphocytes (PBL) and erythrocytes were immediately isolated by density gradient with Ficoll-Paque Plus (Pharmacia, Sweden).

Isolated red blood cells were diluted 50-fold in RPMI 1640 and incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C in the presence and absence of LA.

Isolated peripheral blood lymphocytes were placed in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum, sodium pyruvate (11 μg/ml), and penicillin (100 units/ml), and streptomycin (100 units/ml). Lymphocytes were pretreated with phytohemagglutinin (2 μg/ml) for four hours for mitogenic stimulation then exposed to the LA and glutamate for an additional 48 hours.

2.4. Sample processing for HPLC measurements

At the end of the experiments, an aliquot of Jurkat, erythrocyte or lymphocyte cell suspension was centrifuged at 400*g* for four minutes to separate cell pellets from the culture medium. For C6 and NB41A3 cells, the culture medium was directly removed; the cells were detached with trypsin-EDTA, and centrifuged at 400*g* for four minutes.

The aliquots of culture medium were then mixed with an equal volume of 6% metaphosphoric acid for cysteine analysis or 6% monochloroacetic acid in 60% methanol and 40% ethanol for DHLA analysis. The cell pellets were resuspended in 0.2 M monochloroacetic acid for GSH analysis, while cells were placed in a solution containing 50% 0.2 M monochloroacetic acid and 50% ethanol for DHLA analysis. All the samples were frozen in liquid nitrogen and stored at −80°C until HPLC analysis.

2.5. HPLC determination of thiols and disulfides

Thiol and disulfide determination were performed using the HPLC electrochemical methods as previously reported by Allison and Shoup [39] with some minor modifications. Immediately before the assay, samples were thawed, vortexed, then centrifuged at 15,000*g* for two minutes. The clear supernatant was removed and injected onto the HPLC. Separation of glutathione was achieved in an Alltech Altima C-18 (150 mm × 4.6 mm, 5 μM) column using a mobile phase containing 99% 0.1 M monochloroacetic acid (pH 3.0) and 1% methanol at a flow rate of 1 ml/min. Separation of cysteine and cystine was obtained in an Alltech Altima C-18 (250 mm × 4.6 mm, 5 μM) column using a mobile phase containing 0.1 M monochloroacetic acid (pH 3.0) run at a flow rate of 1 ml/min. HPLC separation of LA and DHLA was achieved using a Rainin Microsorb C-18 (10 cm × 4.6 mm, 3 μM) column. The mobile phase was composed of 0.1 M monochloroacetic acid (50% water, 30% methanol, 20% acetonitrile) and ran at a flow rate of 1 ml/min [40].

2.6. Flow cytometry assay of total cellular thiols

The cellular content of total thiols was measured in Jurkat cell lines and lymphocyte cell suspensions by flow cytometry [41]. Monobromobimane (MBB) was used as the thiol specific fluorescent probe [42].

After treatment, aliquots of the cell suspensions were centrifuged at 400 g for four min. The cell pellets were resuspended in PBS at a final concentration of 10^6 cells/ml and exposed to MBB (40 μM final concentration) for 15 min. Cells were then excited with a 350 nm UV line of an argon-ion laser in a flow cytometer (Epics Elite, Coulter, FL) and the 450 nm emission was recorded as described by Sen [43]. Data was collected from 10,000 viable cells.

2.7. Flow cytometry assay of cell viability

Plasma membrane integrity of PBL was determined flow cytometrically using the non-permeant DNA intercalating dye propidium iodide (Molecular Probes, Eugene, OR) that is generally excluded by viable cells. A 15 mW powered argon-ion laser was used for excitation at 488 nm and an emission signal was collected at 575 nm. Propidium iodide negative cells were interpreted as viable cells.

2.8. Determination of uptake of radiolabeled cystine

The uptake of radiolabeled [^{35}S] cystine was measured as previously described by Novogrodsky [44]. Radiolabeled cystine (1 μCi/ml) was added to RPMI 1640 containing 10^7 cells and incubated in a water bath at 37°C throughout the experiment. After 30 min, the cell suspensions (100 μl) were placed in tubes containing 100 μl of mineral oil and dibutyl phthalate (15 : 85 by volume) by the method described in Ishii [45]. The tubes were spun down (6000*g* for 30 s) and the ends cut. The cut ends were placed in a scintillation vial with 0.9 ml of 0.1 M NaOH. The samples were left overnight in the sodium hydroxide solution. 14 ml of scintillation fluid was added and uptake was read using a Beckman LS 5000 TD scintillation counter. As a measure of background, uptake studies were performed with cells on ice.

2.9. Determination of γ-glutamylcysteine synthetase mRNA expression

Following treatments with LA, cellular pellets were collected as described above and total RNA extracted from cultured cells following the method of Chomczynski and Sacchi [46]. RNA was reverse transcribed, and the subsequent cDNA was amplified using γ-glutamylcysteine synthetase (γ-GCS) primers (5′-ACGAGGCTGAGTGTCCGTCT-3′, 5′-GGTTTGACCTCGGGCAGTGT-3′), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers (5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′, 5′-CATGTGGGCCATGAGGTCCACCAC-3′) which correspond to the human γ-GCS cDNA [47] and human GAPDH cDNA [48], respectively. RT-PCR was performed using a RNA PCR kit (Perkin Elmer, Branchburg, NJ) and carried out for 35 cycles (1 min at 95°C, 1 min at 60°C, 1.5 min at 72°C). The final PCR reaction product was electrophoresed in a 1.7% agarose gel containing 0.2 μg/ml ethidium bromide. PCR bands were quantified using a laser densitometer linked to a computer analysis system (NIH image).

2.10. Statistics

Comparisons were carried out using the Student t-test. A difference was considered to be significant when $p < 0.05$.

3. Results

3.1. Lipoic acid induces cellular GSH increase in many cell types

In confirmation and extension of our earlier work [25], we found that LA in the range of 10–100 μM induces a substantial GSH increase not only in cultured human Jurkat T cells, but in human red blood cells, the C6 glia cell line, NB41A3 neuroblastoma line, and human peripheral blood lymphocytes.

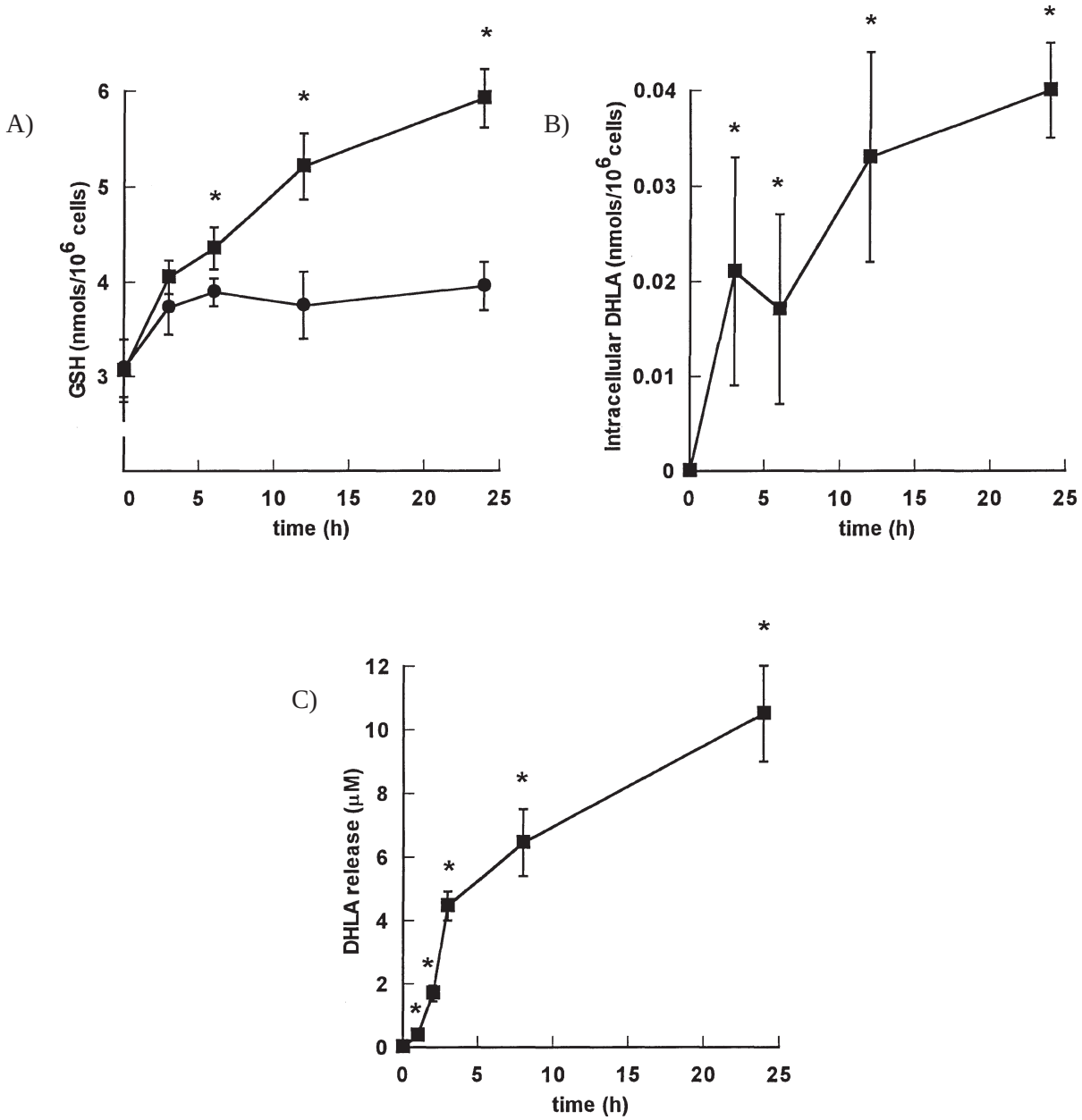


Fig. 1. Increase of GSH after lipoic acid treatment coincides with a build up of intra- and extracellular DHLA. Jurkat cells were incubated in fresh RPMI 1640 with 10% serum, with or without LA (100 μM) for 24 hours. (A) shows the time course of cellular GSH levels of control (●) and LA (■) treated cells. (B) demonstrates the build up of intracellular DHLA after LA treatment. Control cells had no detectable levels of intra- or extracellular DHLA. (C) shows a build up of extracellular DHLA in the medium (cystine free RPMI 1640 with 1% serum) that occurs when cells are treated with LA. Cystine free RPMI 1640 with 1% serum was used to demonstrate DHLA release from Jurkat cells because DHLA is unstable in standard RPMI 1640 with 10% serum because of its high disulfide content. Values are expressed as mean ± standard deviation of three to six experiments. (A) $^*p < 0.05$ vs. control. (B and C) $^*p < 0.05$ vs. time 0.

Table 1
Effect of lipoic acid treatment on cellular glutathione

Cell type	Lipoic acid (μM)	Glutathione increase (%)
Jurkat	10	16.2 ± 13.4
	50	$24.9 \pm 8.7^*$
	100	$49.7 \pm 10.3^*$
C6	10	2.8 ± 13.6
	50	$14.4 \pm 6.1^*$
	100	$28.8 \pm 15.2^*$
NB41A3	100	$37.0 \pm 13.1^*$
Erythrocytes	10	$13.5 \pm 5.1^*$
	50	$38.6 \pm 8.2^*$
	100	$33.6 \pm 12.8^*$
Peripheral blood lymphocytes	100	$48.0 \pm 16.9^*$

Cells were incubated with various concentrations of LA for 24 hours as described in Section 2. PBL were incubated with LA for 48 hours. Glutathione content is expressed as percentage with respect to untreated cells. Values are expressed as mean $\pm$ SD, $n = 3{-}6$; $^*p < 0.05$ vs. control.

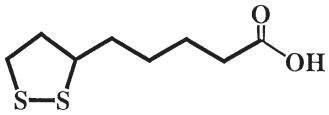
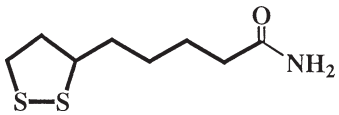
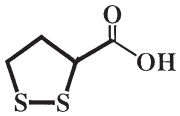
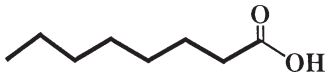
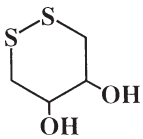
The augmentation of GSH in the different cell types was similar in extent, concentration dependency (Table 1) and time course (data not shown, see Fig. 1A as an example). At higher LA concentrations ($>$ 500 μM; data not shown), greater than levels which can be achieved *in vivo*, LA treatment resulted in some cytotoxic effects [43]. We therefore investigated LA-induced GSH augmentation at the low dosage range (10–100 μM), which is still higher than plasma levels determined after oral application of 300 mg in humans (low μM range [49]), but close to plasma concentration after administration of 10 mg/kg LA in rats [50].

The ability of LA to induce GSH augmentation in Jurkat cells not only occurred in the naturally occurring R-α-enantiomer, S-α-LA and S,R-α-LA as well as lipoamide, and the potential metabolite tetranor (S,R)-LA were equally efficient. In contrast, octanoic acid, identical to LA in its carbon moiety but lacking the dithiolane ring, was inactive, as was the structurally unrelated cyclic disulfide, oxidized dithiothreitol (Table 2).

3.2. LA-induced GSH increase depends on metabolic conversion of LA to DHLA

Both enantiomers of LA are reduced to DHLA in a variety of tissues at the expense of NADH and/or NADPH [36,51]. Glutathione reductase, cytosolic and mitochondrial, has been suggested as the potential LA-reducing enzyme [52], but NADH (instead of NADPH) consumption observed upon LA exposure in heart, liver and kidney suggests alternate pathways [51]. As previously reported [25] the augmentation of GSH (Fig. 1A) in Jurkat T cells exposed to 100 μM R,S-LA parallels the formation of intracellular DHLA (Fig. 1B). When the cells were grown in a medium low in disulfides (cystine free, 1% serum), DHLA could also be detected in the medium, appearing with a similar time course (Fig. 1C). In parallel experiments with normal serum and cystine-containing medium, DHLA was not detectable (data not shown). The DHLA/LA redox potential has been estimated at -0.32 V, which is more negative than the potential of cysteine/cystine (-0.22 V), and therefore DHLA has been demonstrated to directly reduce cystine to cysteine [53]. In fact, DHLA proved unstable in a normal medium, which suggests that released DHLA is instantly oxidized by cystine and protein

Table 2
Effect of lipoic acid and related compounds on cellular GSH levels

Compound		GSH increase (%)
α-lipoic acid 	R-lipoic acid	$51.5 + 7.1^*$
	S-lipoic acid	$46.7 + 11.2^*$
	R,S-lipoic acid	$49.7 + 10.3^*$
Lipoamide 		$42.0 + 7.0^*$
Tetranorlipoic acid 		$60 + 14.1^*$
Octanoic Acid 		$-2.1 + 3.2$
Oxidized Dithiothreitol (DDT) 		$-7.9 + 14.8$

Jurkat cells were treated with the various compounds (100 μM) for 24 hours. Glutathione content is expressed as percentage with respect to untreated cells. Values are expressed as means $\pm$ SD, $n = 3$; $^*p < 0.05$ vs. control.

serum disulfides with formation of cysteine and reduced proteins. Figure 2 shows that cysteine is indeed formed in the medium of Jurkat cells (Fig. 2A) and erythrocytes (Fig. 2B), when incubated with LA.

Based on these data, we reasoned that the LA effect might be mediated by metabolic reduction of LA to DHLA, release of DHLA, followed by chemical reduction of cystine to cysteine in the medium, which then can be taken up (bypassing the weak x_c^- system) and used for GSH synthesis. In order to test this model three types of experiments were performed: i) The time course of GSH augmentation induced by LA and DHLA was compared. Since DHLA does not require previous metabolic reduction, which takes place, e.g., in Jurkat cells at a rate of 0.5 nanomoles/10^6 cells when treated with 100 μM LA, DHLA should induce GSH augmentation more quickly than LA.

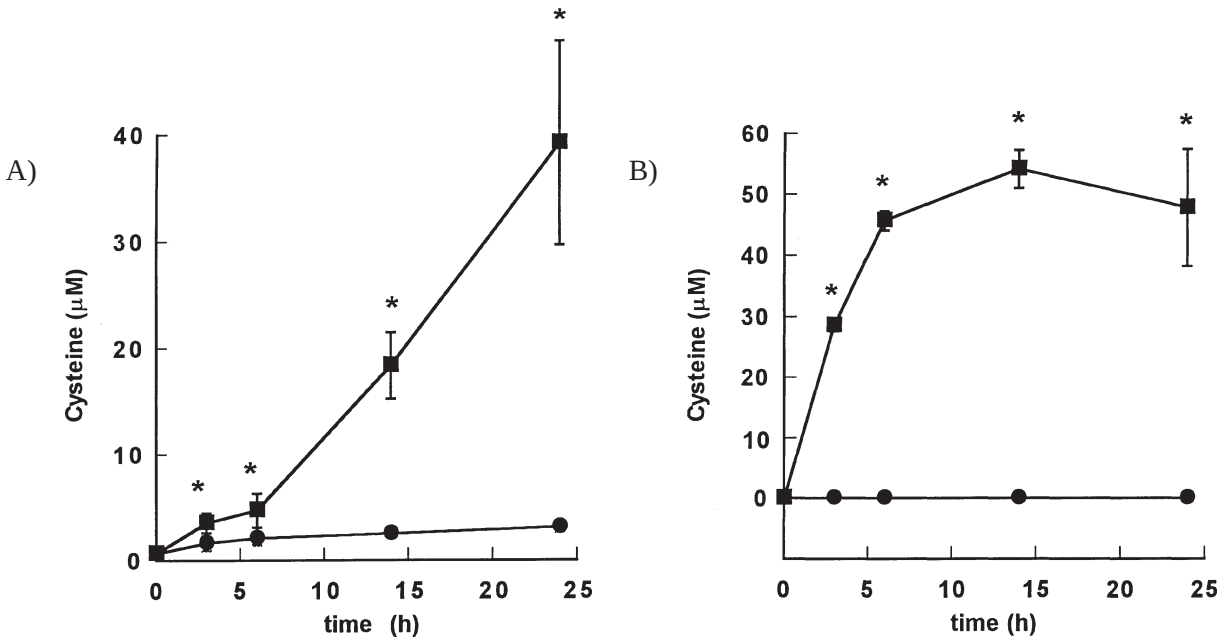


Fig. 2. Cysteine formation in a medium of cultured Jurkat cells (A) and human red blood cells (B) after LA treatment. (Control (●); LA treatment (■)). Jurkat cells (9×10^5 cells/ml) were incubated in RPMI 1640 with 10% serum in the presence and absence of LA (100 μM). Red blood cells (9×10^9 cells/ml) were incubated with or without LA (100 μM) in RPMI 1640. At different time points, cell suspensions were removed, and the medium separated from cells by centrifugation (400 g for four min). The medium was mix with an equal volume of metaphosphoric acid (6%) and analyzed by HPLC. Values are expressed as mean $\pm$ standard deviation of three experiments. $^*p < 0.01$ vs. control.

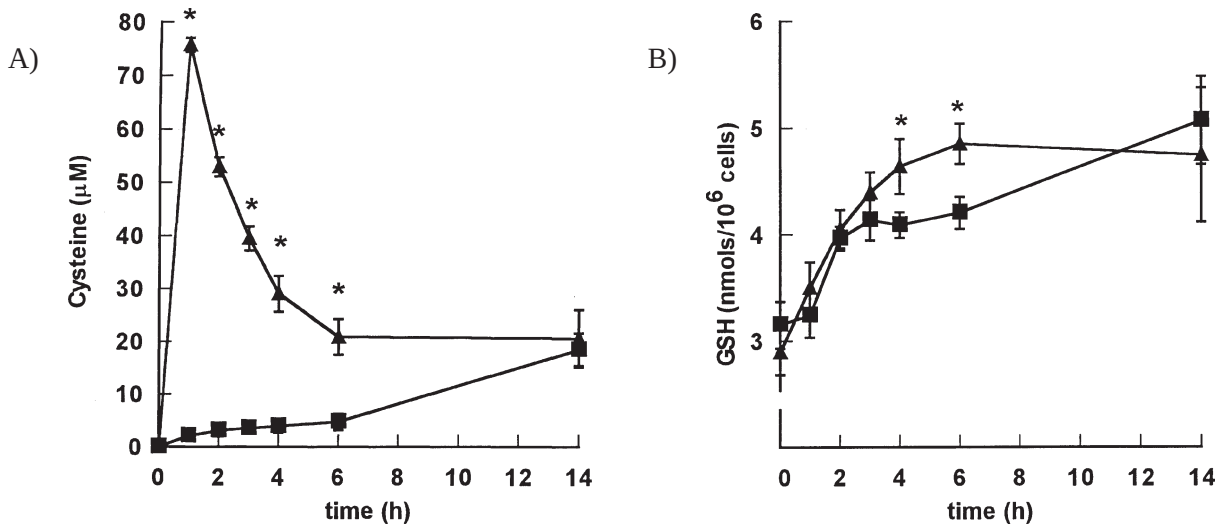


Fig. 3. DHLA causes a rapid formation of cysteine in medium and a faster GSH increase than LA. Jurkat cells were incubated with DHLA (100 μM, ▲) or LA (100 μM, ■) for up to 12 hours. (A) shows cysteine levels in the medium after LA and DHLA treatment. (B) demonstrates cellular GSH levels after LA and DHLA treatment. Values are expressed as mean $\pm$ standard deviation of four experiments. $^*p < 0.05$ vs. LA.

ii) The uptake of [^{35}S] cystine was measured. A faster utilization of radiolabeled cystine in LA-treated cells should occur since cysteine is generated and the uptake of cysteine is tenfold faster than that of cystine [38]. The removal of the catalytic redox couple LA/DHLA by changing the medium should decrease cystine utilization. iii) Inhibition of LA reduction (e.g., by blocking NADPH supply by glucose 6-phosphate dehydrogenase (G-6-PDH)) should reduce the GSH augmentation by LA. Figure 3A demonstrates that addition of DHLA directly into the medium, through direct reduction of cystine to cysteine, generates large concentrations of cysteine in the medium, almost instantaneously. GSH augmentation is therefore faster when Jurkat cells are exposed to DHLA instead of LA (Fig. 3B). As anticipated, a trend towards faster repletion of the cellular GSH by DHLA is observed from the beginning, but the difference in GSH between LA- and DHLA-treated cells only becomes significant beyond the third hour of incubation because of the time required for GSH biosynthesis. However, as cysteine levels in the medium become similar, cellular GSH levels become almost equivalent. Figure 4 demonstrates that LA increases the utilization of [^{35}S] cystine, presumably by the DHLA reduction of [^{35}S] cystine to [^{35}S] cysteine and consequent uptake by the ASC system. The effect of LA on [^{35}S] cystine utilization is abolished by exchange of medium (washing of LA treated cells), indicating LA/DHLA must be present in the medium to shuttle reduction equivalents across the cellular membrane to increase cystine utilization. Dehydroepiandrosterone, which has been shown to inhibit G-6-PDH [54], decreases reduction of LA to DHLA by more than 90% (data not shown) and significantly inhibits cysteine formation in the medium of LA treated cells (Fig. 5A). The inhibition of cysteine formation results in a decrease of GSH augmentation by LA in Jurkat T cells (Fig. 5B), whereas mercaptoethanol-induced GSH augmentation remained unaffected. The partial suppression of the LA-effect by DHEA might be explained by a G-6-PDH-independent pathway for reducing LA. Taken together these experiments clearly reveal that LA-induced GSH augmentation depends on the reduction of LA to DHLA and consequent cysteine formation in the medium.

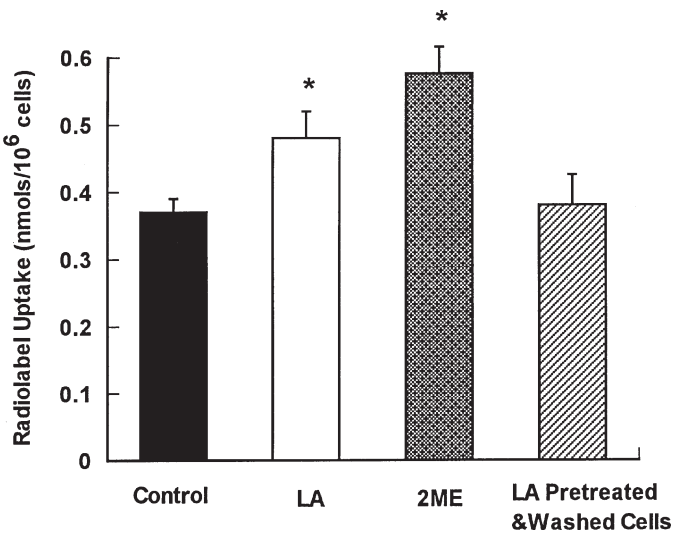


Fig. 4. Uptake of [^{35}S] radiolabeled cystine in Jurkat cells. 1 μCi/ml of radiolabeled cystine was added to RPMI 1640 (containing 265 μM cystine). Jurkat cells (10^7 cells) were treated with LA (100 μM) and 2-mercaptoethanol (100 μM) immediately after addition of radiolabeled tracers. LA pretreated and washed cells were incubated with LA (100 μM) for 12 hours, washed with PBS, and immediately placed in RPMI 1640 with radiolabeled cystine. The cells were incubated for 30 min with [^{35}S] cystine. Values are expressed as mean $\pm$ standard deviation of three experiments. $^{*}p < 0.05$ vs. control.

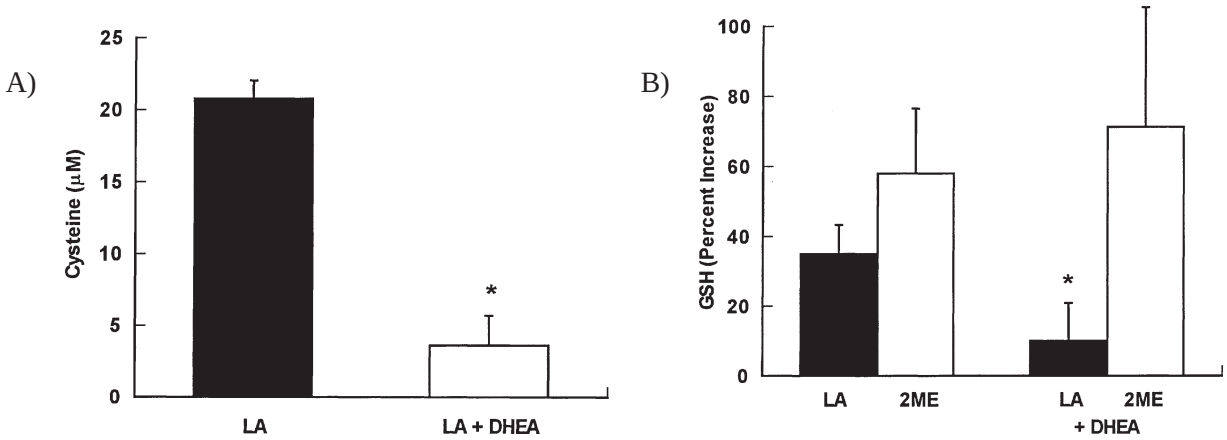


Fig. 5. Inhibition of LA reduction by DHEA decreases LA-induced cysteine formation and GSH augmentation. DHEA, an inhibitor G-6-PDH, slows down reduction of LA to DHLA. Jurkat cells were treated with lipoic acid (100 μM) in the presence and absence of DHEA (150 μM) for 12 hours. (A) shows cysteine levels in the medium of Jurkat cells after treatment. (B) demonstrates the effect of DHEA on LA-induced GSH augmentation. (B) is expressed as percentage increase with respect to control. Values are expressed as mean $\pm$ standard deviation, $n = 4$; $^{*}p < 0.05$ vs. cells not treated with DHEA.

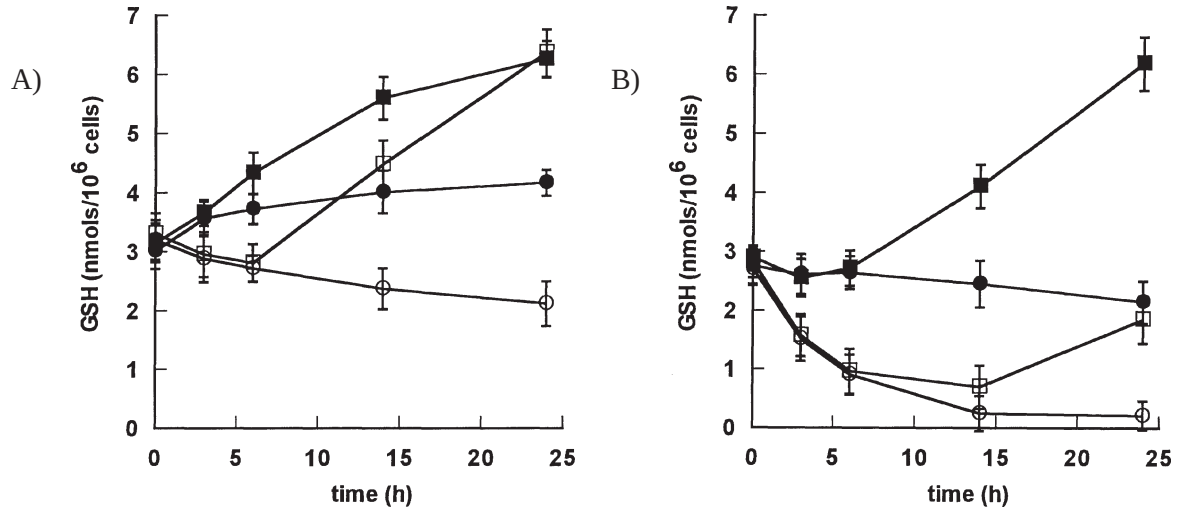


Fig. 6. LA-induced GSH increase can bypass glutamate inhibition of the x_c^- cystine transport system. (Control (●), LA (■), glutamate 5 mM (○), LA 100 μM + glutamate 5 mM (□).) (A) Jurkat cells were incubated with LA and glutamate in normal RPMI 1640 (265 μM cystine). (B) Jurkat cells were treated with glutamate and LA in RPMI 1640 with low cystine levels (65 μM) ($n = 3$).

3.3. LA-induced GSH augmentation is insensitive to glutamate

Treatment of Jurkat cells with glutamate, a competitive inhibitor of the x_c^- transporter, inhibits cystine uptake and consequently lowers cellular GSH levels. A 50% loss of GSH is experienced in Jurkat cells incubated with glutamate (5 mM) in standard medium (265 μM cystine; Fig. 6A). When Jurkat cells were incubated with glutamate (5 mM) in low cystine (65 μM) medium, a 93.5% loss of GSH occurred, indicating an almost complete inhibition of cystine utilization (Fig. 6B). At high cystine concentrations, 100 μM LA not only completely prevented the glutamate-dependent GSH loss

but increased the GSH content to levels above control despite the presence of excessive glutamate concentrations (Fig. 6A). Even at a low cystine concentration (Fig. 6B), LA could compensate for the glutamate-induced fall in cellular GSH. Since at low cystine and 5 mM glutamate, cystine uptake via the x_c^- transporter was almost completely blocked, LA-induced cysteine uptake via the ASC system appears to fully account for the LA-facilitated GSH synthesis.

3.4. LA-induced GSH augmentation requires function but not an induction of γ-glutamylcysteine synthetase

Cellular GSH in Jurkat cells is rapidly depleted by the inhibitor of γ-glutamylcysteine synthetase, BSO, and this decline is not prevented by LA (Fig. 7). This indicates that maintenance of the GSH status depends on its continuous *de novo* synthesis. Also, the failure of LA to prevent the BSO-induced GSH loss shows that the effect of LA on GSH levels requires a functioning γ-glutamylcysteine synthetase.

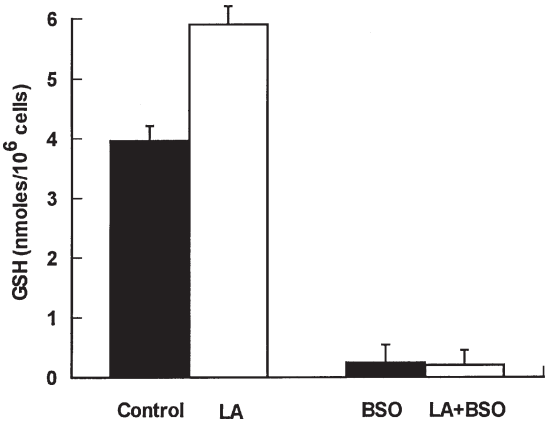


Fig. 7. Dependence of GSH augmentation by LA on *de novo* synthesis of GSH. Jurkat cells were treated with lipoic acid (100 μM) in the presence and absence of BSO (200 μM), an inhibitor of γ-glutamylcysteine synthetase, for 24 hours ($n = 3$).

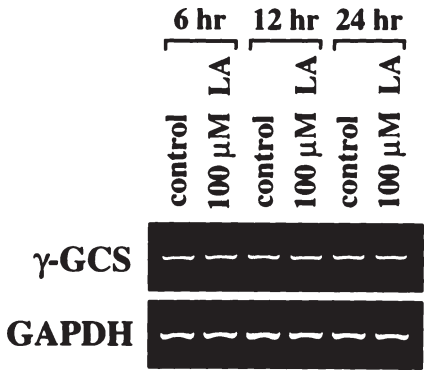


Fig. 8. The effect of LA treatment on γ-glutamylcysteine synthetase-mRNA expression. Jurkat cells were treated with LA (100 μM) for various time points and the mRNA levels of γ-GCS was determined by RT-PCR. GAPDH, a constitutively expressed enzyme, served as an internal control. PCR bands, read using a laser densitometer, showed no changes in mRNA levels of γ-GCS.

With regard to recent reports on the inducibility of γ-glutamylcysteine synthetase [55], we also monitored the pertinent mRNA levels by RT-PCR in comparison to GAPDH as a constitutively expressed enzyme. As is shown in Fig. 8, LA does not affect γ-glutamylcysteine synthetase mRNA levels. This data is in agreement with our previously reported lack of influence by protein synthesis inhibitors on LA-dependent GSH augmentation [25].

3.5. Influence of LA on heterogeneous cell populations as evidenced by flow cytometric analysis

When cellular thiols in conventionally grown Jurkat cells were labeled with monobromobimane (MBB) and subjected to flow cytometric analysis, the cellular fluorescence intensities did not appear completely normally distributed. Instead, a very small subpopulation of cells (2%) exhibiting a fluorescence intensity of less than 10% of the main cell population was detectable (Fig. 9A). Treatment of Jurkat cells with LA (100 μM) for 24 hours increased the average fluorescence and practically eliminated the low fluorescence cells ($<$ 1%), presumably by normalizing their thiol status (Fig. 9B), as is evident from a 50% average increase of GSH concentration (Table 1). The heterogeneity in thiol status was even more obvious in mitogen-stimulated peripheral blood lymphocytes (Fig. 10A). A substantial proportion of the cells (8%) exhibited a MBB-fluorescence around 1% of 'normal', whereas cells of medium fluorescence were rare. HPLC analysis shows that treatment of PBL with LA increase cellular GSH by 48% (Table 1). Flow cytometric analysis of PBL treated with 100 μM LA showed that LA mainly eliminated the 'low thiol' subpopulation, and increased the average MBB-fluorescence (Fig. 10B). Inhibition of cystine uptake by 5 mM glutamate in PBLs (Fig. 10C) clearly creates two distinct subpopulations of cells showing either normal (84%) or extremely low MBB-fluorescence (16%) leaving almost none of the cells in the intermediate fluorescence range. In the presence of 100 μM LA, 5 mM glutamate did not augment the low fluorescence population. Instead an almost homogeneous high fluorescence cell population was obtained (Fig. 10D). Flow cytometric analysis of cell viability using propidium iodide showed that in all treatments the cell viability was above 95% (data not shown). It is therefore unlike that the disappearance of the low fluorescence cell population after lipoic acid treatment was a result of cell death but rather due to a normalization

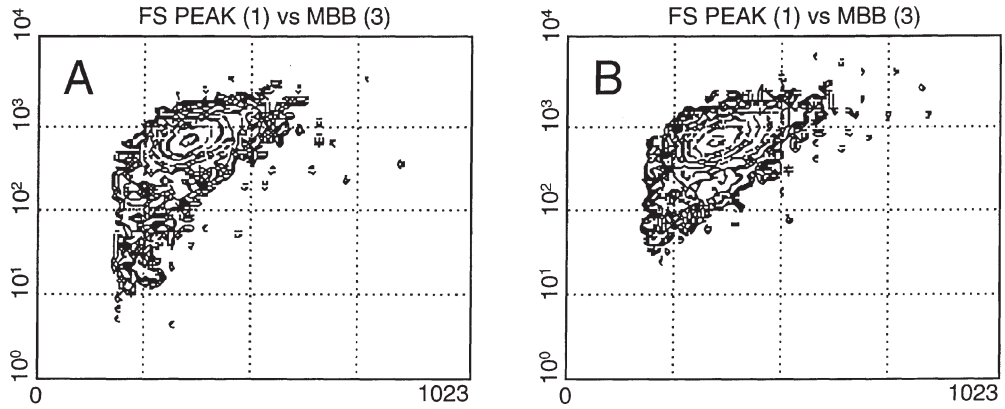


Fig. 9. Flow cytometric analysis of Jurkat cells for cellular thiol content. The x-axis represents forward scatter signal or cell size. The y-axis is a log scale representing MBB signal from UV-excited cells. Jurkat cells, after treatment with or without LA, were labeled with monobromobimane (40 μM) for 15 min and then excited with a 350 nm UV line of an argon-ion laser in a flow cytometer and the 450 nm emission was recorded. Data are displayed as contour plots. (A) Control and (B) LA (100 μM, 24 h) treated cells are shown.

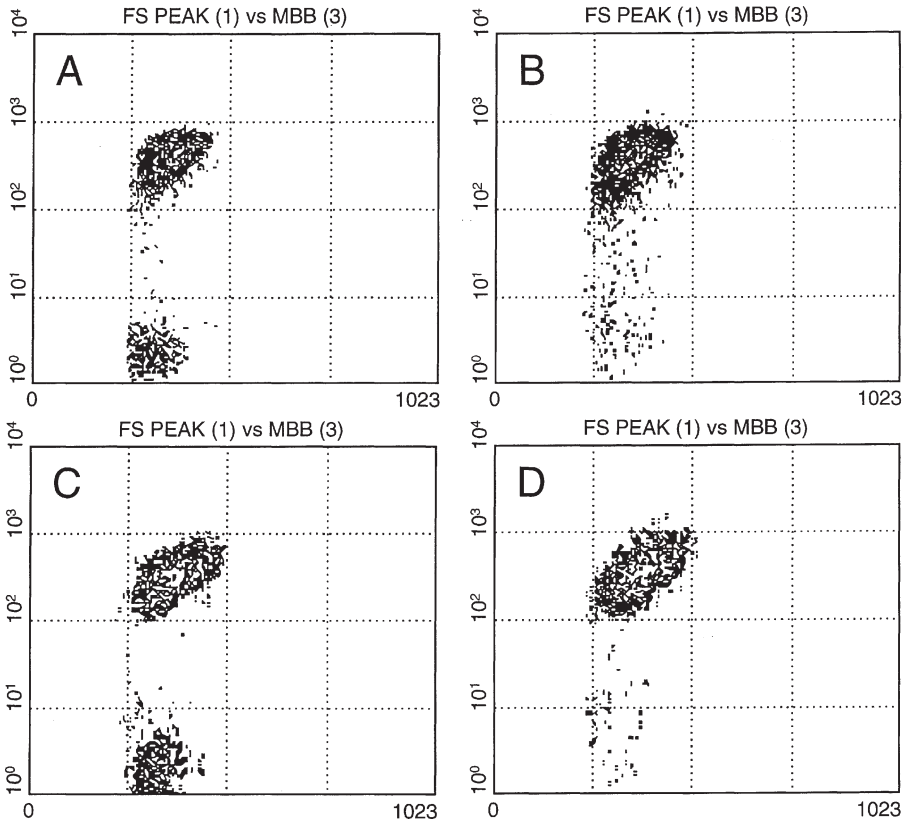


Fig. 10. Flow cytometric analysis of human peripheral blood lymphocyte cellular thiol content. The x-axis represents forward scatter signal or cell size. The y-axis is a log scale representing the MBB signal from UV-excited cells. PBL were mitogenically stimulated with phytohemagglutinin (2 μg/ml) and incubated with or without LA and glutamate for 48 hours. After treatment, PBL were labeled with monobromobimane (40 μM) for 15 min and then excited with a 350 nm UV line of an argon-ion laser in a flow cytometer and the 450 nm emission was recorded. (A) A dot plot analysis of untreated PBL. A subpopulation (8% of the total population) of PBL with extremely low thiol content (1% fluorescence of the average population) can be seen. (B) Treatment of PBL with LA (100 μM) creates an upward shift in average fluorescence and reduces the population of low thiol PBL to less than 1%. (C) Treatment of PBL with glutamate (5 mM) negatively alters thiol status, resulting in a larger subpopulation (16%) of extremely low MBB fluorescent cells. (D) PBL treated with glutamate (5 mM) and LA (100 μM). LA treatment results in a virtual elimination of the low thiol subpopulation (less than 1%) that is enhanced after glutamate treatment and results in an increase in the mean fluorescence of the cell population.

of cellular thiol status. These observations indicate that LA-induced GSH augmentation and related normalization of cellular thiol status is most pronounced in particularly compromised cells, but does not increase cellular thiol content beyond a defined limit (compare Figs 9A and B and 10A, B, and C, D respectively).

4. Discussion

Our data demonstrate that LA effects GSH levels by shuttling cellular reducing equivalents across the membrane to the extracellular environment (Fig. 11). LA is taken up by cells, metabolically reduced to DHLA by NADH and NADPH utilizing pathways, and released into the medium. DHLA subsequently reduces cystine to cysteine, whereupon cysteine is taken up by the systems for transport

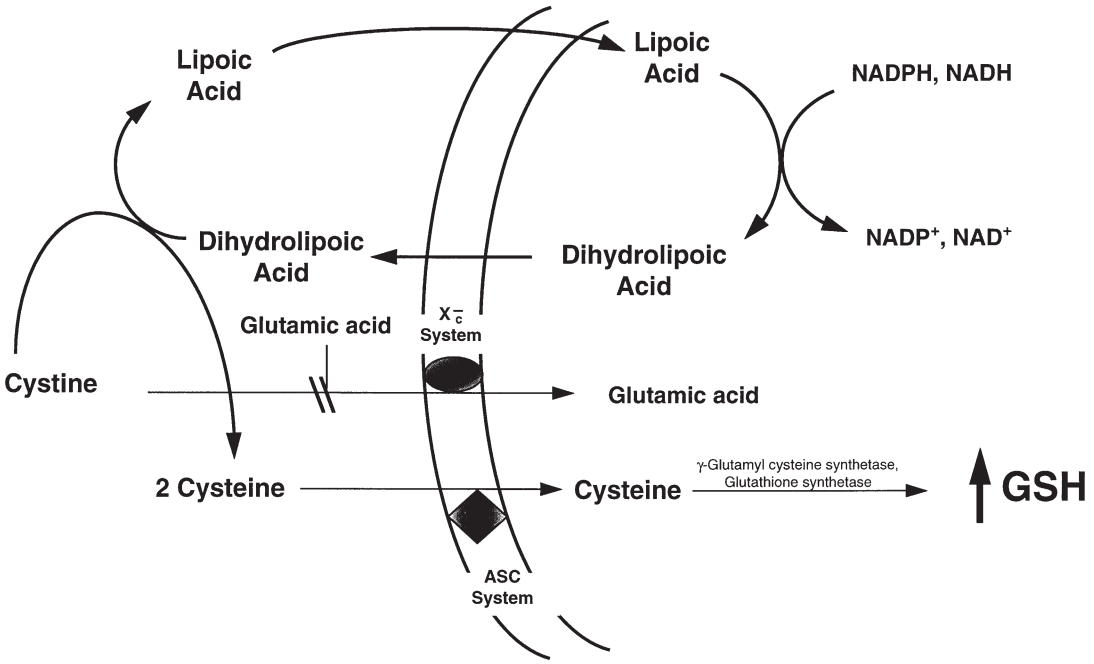


Fig. 11. Schematic outline of the proposed mechanism by which lipoic acid increases cellular GSH levels. Lipoic acid enters cells, is reduced to DHLA, and released into the medium. DHLA subsequently reduces cystine to cysteine. The newly formed cysteine is taken up by the ASC transporter, thereby decreasing the cell's dependence on cystine uptake by the glutamate-sensitive x_c^- transport system. The efficient cysteine uptake by the ASC transport system leads to an increase of the influx of cysteine, the rate limiting substrate in GSH synthesis, resulting in an elevation of cellular GSH levels.

of neutral amino acids. These comprise the systems ASC, A and L, with the ASC system being the most relevant one (for a review see Bannai [18,19]). Through this 'redox shuttle', lipoic acid treatment can increase cystine utilization for synthesis of GSH.

The advantage of channeling cystine, via reduction, through the ASC system instead of relying on the x_c^- system for direct transport of cystine is explained by the absence or low efficiency of the later transporter in many cell types (erythrocytes, splenic lymphocytes, lymphoma cells, Ehrlich ascites cells) and its susceptibility to inhibition by glutamate [18]. The importance of the neutral amino acid transport system versus direct cystine uptake was first deduced from the requirement of cultured mouse lymphoma cells (L1210) for mercaptoethanol by Ishii [56], who showed that cystine reacts with 2-mercaptoethanol to yield a mixed disulfide taken up via the leucine transporter. But Gmünder et al. [38], by measuring transport activities directly, demonstrated that the x_c^- system is also extremely weak in mitogen-stimulated peripheral blood lymphocytes and a variety of T cell lines, whereas the ASC system proved at least ten times as efficient. It therefore may be tentatively concluded that providing cysteine by means of the ASC system is crucial at least for some types of cells.

Certainly, deficiencies in cystine uptake are more easily detected under tissue culture conditions than *in vivo*. Due to its tendency to auto-oxidize, cysteine is not present in conventional tissue culture media for longer than 30 min, whereas *in vivo*, between 10% and 20% of total cysteine is reduced under normal conditions. Still, the normal plasma cysteine concentrations of 10–30 μM are in the range of reported Km values for the ASC system [18] and any increase of plasma cysteine will yield higher cysteine uptake. Also, the extracellular cystine/cysteine ratio will be increased at least locally during host defense or inflammatory reactions associated with the respiratory burst, and cells

depending on the ASC system must become compromised. In addition, cells having the x_c^- transport system will benefit from reduction of extracellular cysteine under conditions where uptake of cystine is impaired by an unfavorable cystine/glutamate ratio, as reported for HIV-infected patients [20,21].

Lipoic acid-facilitated cystine utilization relates to cellular GSH content in a straightforward manner. Glutathione, due to GSH and GSSG export, is lost from all tissues although with tissue-specific rates [1,10]. Maintenance of cellular glutathione therefore requires *de novo* synthesis, commonly exerted by γ-glutamylcysteine synthetase and glutathione synthetase. Synthesis of GSH is regulated at the key enzyme of the two-step pathway, i.e., γ-glutamylcysteine synthetase. Induction of γ-glutamylcysteine synthetase, characterized as transcriptional gene activation, has been reported to occur in pulmonary endothelial cells exposed to redox-cycling quinones [55]. γ-Glutamylcysteine synthetase may be down-regulated by glucagon and phenylephrine via phosphorylation [57], is feedback inhibited by GSH [1], and depends on cellular cysteine levels which are close to Km values [1]. Our data rule out any inductive effect of LA, since the LA induced GSH increase was unaffected by inhibition of protein synthesis [25] and levels of γ-glutamylcysteine synthetase mRNA remained unchanged after LA treatment (Fig. 8). Also, GSH augmentation by LA in erythrocytes, which lack protein synthesis (Table 1), rules out the necessity of enzyme induction. There is neither a rationale nor any experimental evidence for interference of LA with the hormone-controlled phosphorylation status of the γ-glutamylcysteine synthetase in the systems investigated. Thus, the correspondence of cysteine appearance in the medium and rise in cellular GSH in terms of time and concentration strongly suggests that increased turnover by γ-glutamylcysteine synthetase due to improved cysteine availability is the main effect of LA.

Addressing the relevance of increased cellular GSH, we are confronted with more serious problems. Uhlig and Wendel [58] pointed out that a drop of cellular GSH to about 10% of normal should not affect cellular function and integrity significantly. This is particularly true for the kinetically well-characterized glutathione-dependent hydroperoxide metabolism [12,59]. At estimated physiological peroxide levels up to 10^{-6} M all types of glutathione peroxidases, the classical type [12], the extracellular type [60] or the phospholipid hydroperoxide glutathione peroxidase [61] should be operating at maximum velocity irrespective of a $\pm$50% variation of cellular GSH. Theoretically, these enzymes are fully reduced within this range of GSH concentration with their active site, selenol, ready to scavenge any hydroperoxide molecule. But such extrapolation from kinetic studies to the *in vivo* situation may be misleading. First of all, the commonly quoted steady state tissue levels of H_2O_2 [62] or lipid hydroperoxides [63] in the low micromolar range may be significantly exceeded locally in pathological situations and under these conditions, GSH concentrations become relevant to glutathione peroxidase rates. Theory also predicts that cellular GSH will not decrease gradually but will drop to practically zero, as soon as the flux of hydroperoxides exceeds the maximum rate of GSH regeneration, which is usually limited by NADPH supply. If persistent over time, such conditions will result in an accelerated loss of total glutathione due to export of GSSG. These considerations suggest that small decrements in average tissue GSH likely reflect a substantial decrease in a corresponding percentage of cells.

This predictable heterogeneity of cellular GSH content is indeed shown to occur by the flow cytometry experiments with normal peripheral blood lymphocytes labeled with monobromobimane (Fig. 10) and even more clearly in lymphocytes of HIV-infected patients [23]. Admittedly, monobromobimane does not label only cellular GSH, but all SH groups including proteins sulfhydryl groups, and ongoing experiments demonstrate that a 90% depletion in cellular GSH by BSO treatment results in a 40% decrease in monobromobimane fluorescence in contrast to a 97% fluorescence decrease in cells in which all SH groups were reacted before with N-ethylmaleimide [43]. However, this consideration

strengthens rather than weakens the relevance of distinct subpopulations observed in peripheral blood lymphocytes exhibiting an MBB fluorescence in the one percentile range. Such cells may indeed have GSH levels close to zero. Interestingly, treatment with glutamate to inhibit GSH synthesis via blockade of x_c^- mediated cystine uptake hardly decreases MBB fluorescence of the main cell population but augments cells with extremely low fluorescence, which again implies that cellular GSH either remains almost normal or becomes extremely compromised.

The effects of LA are thus perhaps not correctly interpreted only in terms of a 20–50% increase of cellular GSH, which would possibly be irrelevant, but rather as a normalization of cellular GSH in a corresponding percentage of previously compromised cells. In this sense, restoration of cellular glutathione may be considered a promising rationale to guarantee adequate functioning of the realm of GSH-dependent cellular reactions.

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