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## RAPID COMMUNICATION

# α-LIPOIC ACID REDUCTION BY MAMMALIAN CELLS TO THE DITHIOL FORM, AND RELEASE INTO THE CULTURE MEDIUM

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**ABSTRACT**-Lipoic acid has been reported recently to be an effective antioxidant in biological systems. It may act *in vivo* through reduction to its dithiol form, dihydrolipoic acid. Using a dual Hg/Au electrode, and HPLC with electrochemical detection, a method was developed which allowed simultaneous measurement of lipoic acid and dihydrolipoic acid, at nanomolar levels. (*RS*)-α-Lipoic acid was added to human cells in tissue culture (Jurkat T-lymphocytes and primary neonatal diploid fibroblasts). Lipoic acid was converted rapidly by the cells to dihydrolipoic acid, which accumulated in the cell pellet. Monitored over a 2-hr interval, dihydrolipoic acid was released, and several-fold more dihydrolipoic acid could be found in the medium than in the pellet.

Key words: lipoic acid, dihydrolipoic acid, thiols, antioxidant, electrochemical detector

α-Lipoic acid (Fig. 1) functions as the prosthetic group for several redox reactions catalyzed by cellular α-keto-acid-dehydrogenases, such as the pyruvate-dehydrogenase complex [1]. To carry out this function, the disulfide group of the lipoic acid dithiolane ring is reduced to its dithiol form, DHLA‡ (Fig. 1).

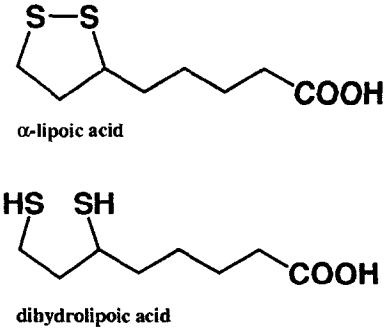


Fig. 1. Oxidized (top) and reduced (bottom) forms of lipoic acid.

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‡Abbreviations: DHLA, dihydrolipoic acid; ECD, electrochemical detector.

DHLA is an excellent reducing agent, and may have the potential to enhance human antioxidant defenses against free-radical mediated pathology. Work from our laboratory has shown that DHLA can scavenge many types of active oxygen species; we have demonstrated the ability of exogenous DHLA to prevent oxidative damage to membranes [2], low-density lipoproteins [3], and the reperfused rat heart [4]. These findings indicate that the *in vivo* conversion of lipoic acid to DHLA might be of considerable importance.

Previous findings suggest that exogenous lipoic acid is reduced to DHLA in biological systems: mitochondria and bacteria [5], perfused rat liver [6], and isolated hepatocytes [7]. In 1989, Peinado and co-workers [6] proposed that the conversion of lipoic acid to DHLA was responsible for the antioxidant properties of lipoic acid.

Since both lipoic acid and DHLA lack a strong UV-absorbing chromophore [8], determination of these compounds by HPLC at trace levels has not been possible with conventional UV detection. Although sensitive analysis of lipoic acid in biological samples can be accomplished with gas chromatography [9], or with gas chromatography-mass spectrometry [10], these methods have not been able to distinguish between the oxidized and reduced forms.

The ECD is a useful alternative for HPLC analysis. Teichert and Preuss [11] measured lipoic acid and DHLA by HPLC with a single glassy-carbon electrode ECD, at the high oxidation potential of 1.1 V. However, very specific HPLC analysis of thiols and disulfides can be accomplished by a dual Hg/Au electrode [12]. We report the first adaptation of the dual Hg/Au electrode ECD to HPLC analysis of lipoic acid and DHLA.

## MATERIALS AND METHODS

(*RS*)-α-Lipoic acid and (*RS*)-DHLA were obtained from ASTA Medica (Frankfurt, Germany). The human T-lymphocyte tumor line, Jurkat cells, was obtained from the American Type Culture Collection, Bethesda, MD, and grown as a suspension culture in RPMI-1640 medium, as described previously [13]. Human primary neonatal fibroblasts were provided by Dr. Alam Hussain, University of California, San Francisco, CA.

HPLC determination of lipoic acid and DHLA was carried out on a Rainin Microsorb 3 μm C18 column, 10 cm x 0.46 cm (Rainin Instruments, Emeryville, CA). The HPLC mobile phase was 50% $H_2O$/30% methanol/20% acetonitrile, with 2% monochloroacetic acid (Fisher, Pittsburgh, PA). The flow rate was 1.0 mL/min, with a 20-μL injection loop.

Measurement of lipoic acid and DHLA in the HPLC column eluant was accomplished with an electrochemical detector (BAS Instruments, West Lafayette, IN), with a dual Hg/Au electrode [12]. The first electrode surface, which reduced lipoic acid to DHLA, was set at -0.875 V. The

second electrode, which monitored the spontaneous chemical oxidation of the DHLA thiol at the Hg surface, was set at +0.05 V.

An 8-mM solution of lipoic acid in medium was mixed with the cell cultures to achieve the target concentrations. For most experiments, we used 10 million Jurkat cells in 10 mL of medium. The culture medium was mixed with an equal volume of methanol, containing 1% monochloroacetic acid and centrifuged, and the supernatant was injected onto the HPLC. The cell pellet was solubilized with 1 mL of the HPLC mobile phase, which disrupts the cell membranes because of its content of methanol and acetonitrile. After centrifugation to eliminate cell debris, the solubilized cell contents were analyzed by HPLC.

# RESULTS

**Formation of DHLA in the cell pellet.** We initially evaluated cells in suspension, both Jurkat cells and recently detached fibroblasts. The HPLC method allows measurement of both lipoic acid and DHLA in the pellet (Fig. 2). Because the dual-electrode detector gives a substantially larger peak area for DHLA than for lipoic acid, the 2.0 nmol of DHLA in this HPLC trace produced a 3-fold larger peak than the 4.3 nmol of lipoic acid also detected in this cell pellet.

When lipoic acid was added to the culture medium without cells present, no formation of DHLA was observed. Within 5 min after adding lipoic acid to the culture medium in the presence of Jurkat cells, the DHLA levels (per volume of packed cell pellet) achieved about 25% of the concentration of lipoic acid in the culture medium. Data for Jurkat cell pellets, at various concentrations of lipoic acid in the medium, are seen in Fig. 3.

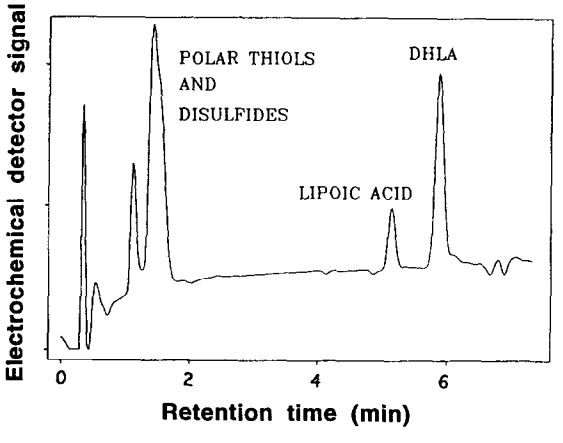


Fig. 2. HPLC analysis of lipoic acid (4.3 nmol) and DHLA (2.0 nmol) in Jurkat cell pellets.

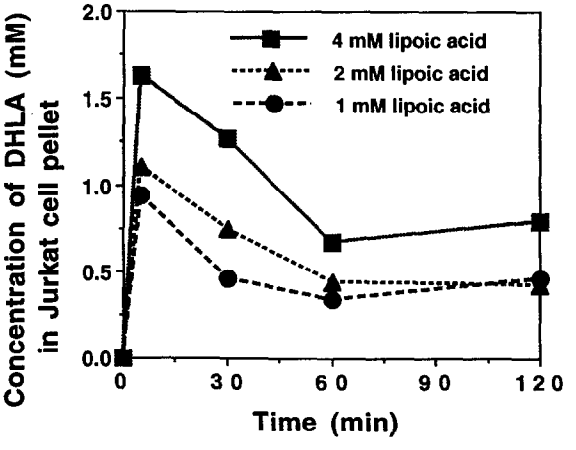


Fig.3. Concentration of DHLA in Jurkat cell pellets after incubation with 1, 2, and 4 mM lipoic acid.

Over the 2-hr interval, there was a decline in the level of DHLA in the pellet from its maximum at 5 min after addition of lipoic acid to the cells.

We hypothesize that intracellular DHLA is oxidized continuously back to lipoic acid. When the initial reservoir of reducing equivalents is depleted from the cell, the cell achieves a steady state, with conversion to DHLA occurring at the same rate as oxidation to lipoic acid.

The volume of the packed cell pellet was estimated after a 10 min spin in a conical centrifuge tube at 2000 x g. In a separate experiment, we calculated from the quantity of pellet-associated Trypan Blue that 50-60% of the pellet volume was culture medium. Therefore, the values in Fig. 3, taken by direct conversion of pellet volume to cytoplasmic volume, underestimate the true cytoplasmic concentration of DHLA by about 50%.

**Levels of lipoic acid in cells and culture medium.** Since a large excess of lipoic acid (1-4 mM) was used for these studies, the total conversion to DHLA did not exceed 2% in any experiment, and the level of lipoic acid did not change appreciably. Over these time intervals (5 min-2 hr) we did not observe formation of shorter chain length β-oxidation metabolites of lipoic acid by these cells.

**Release of DHLA by cells.** In all experiments, the Jurkat cells and fibroblasts released DHLA into the culture medium. The concentration of DHLA in the Jurkat cell medium is shown in Fig. 4, for several levels of lipoic acid in the culture medium. As with the pellet, there was a slow decline in the concentration of DHLA in the medium. This may be due to decomposition of DHLA in the culture medium, as discussed below.

At all time points, the total quantity of DHLA in the culture medium was greater than the quantity of DHLA in the cell pellet (although the concentration of DHLA was much higher in the pellet than in the medium). The distribution of total DHLA between the culture medium and the pellet for Jurkat cells incubated with 2 mM lipoic acid is shown in Fig. 5.

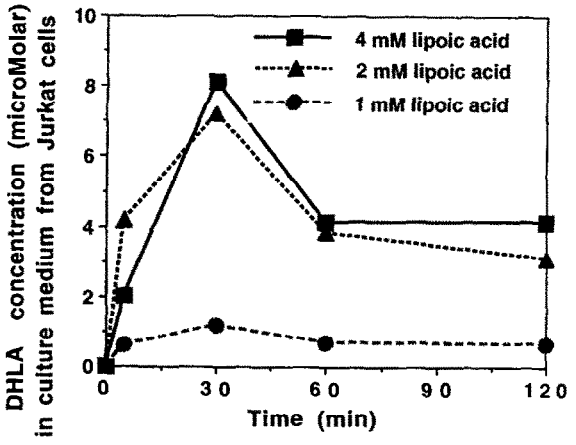


Fig. 4. Concentration of DHLA in the Jurkat cell medium after incubation with 1, 2 and 4 mM lipoic acid.

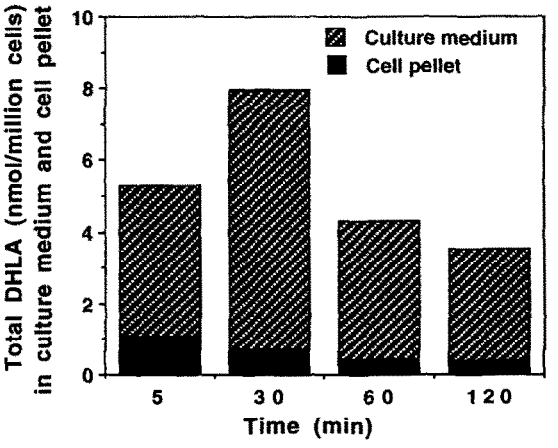


Fig. 5. Total quantity of DHLA in the culture medium and the cell pellet of Jurkat cells incubated with 2 mM lipoic acid.

The release of DHLA by anchored fibroblasts at confluence is shown in Fig. 6. The amount released was much greater than from detached fibroblasts (data not shown), suggesting that anchorage of the fibroblasts supports their active metabolism and reducing activities.

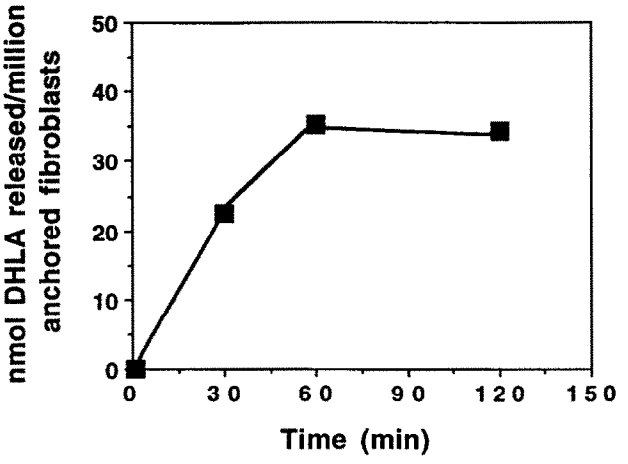


Fig. 6. DHLA released into the medium by anchored primary human neonatal fibroblasts incubated in 4 mM lipoic acid.

**Stability of DHLA in culture medium.** We added DHLA directly to RPMI-1640 medium (incubated at 37°, in 95% air/5% $CO_2$), and determined its rate of decomposition. In several experiments, with DHLA concentrations of 20 and 200 μM, we found that the half-life for DHLA decay in RPMI-1640 medium was 10 min (+/- 5 min). This rapid degradation was brought about by some constituents of the culture medium, because virtually no breakdown of DHLA (<5%/hr) was seen in 10 mM $NaPO_4$ buffer, pH 7.0, at 37°. Because of this continual decomposition of DHLA in the culture medium, the true amount of DHLA released by cells was substantially greater than the quantities estimated from Figs. 4 and 6.

## DISCUSSION

These findings confirm and extend other evidence that lipoic acid is readily accumulated and converted to DHLA in cellular systems. In recent years, several observations have indicated that exogenous oral doses of lipoic acid (which exceed the amounts obtained from cellular biosynthesis by several orders of magnitude) can exert antioxidant effects *in vivo*. In recent work from our laboratory, lipoic acid in the medium of Jurkat cells prevented the activation of NF-κB by TNF-α [13]. Because similar results have been obtained with *N*-acetylcysteine, which boosts cellular levels of glutathione [14], it was thought that the effects of lipoic acid on NF-κB expression are mediated through the reduction of lipoic acid to DHLA.

Our findings indicate that normal mammalian cells are capable of reducing lipoic acid to DHLA, and secreting DHLA in quantity. Since DHLA is released by cells, the role of DHLA in extracellular fluids needs further investigation.

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