Free Radical Biology & Medicine, Vol. 33, No. 1, pp. 83–93, 2002

0891-5849/02/$–see front matter

PII S0891-5849(02)00862-6

Original Contribution

UPTAKE, RECYCLING, AND ANTIOXIDANT ACTIONS OF α-LIPOIC ACID IN ENDOTHELIAL CELLS

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(*Received* 5 *March* 2002; *Revised* 4 *April* 2002; *Accepted* 4 *April* 2002)

ABSTRACT—α-Lipoic acid, which becomes a powerful antioxidant in its reduced form, has been suggested as a dietary supplement to treat diseases associated with excessive oxidant stress. Because the vascular endothelium is dysfunctional in many of these conditions, we studied the uptake, reduction, and antioxidant effects of α-lipoic acid in cultured human endothelial cells (EA.hy926). Using a new assay for dihydrolipoic acid, we found that EA.hy926 cells rapidly take up and reduce α-lipoic acid to dihydrolipoic acid, most of which is released into the incubation medium. Nonetheless, the cells maintain dihydrolipoic acid following overnight culture, probably by recycling it from α-lipoic acid. Acute reduction of α-lipoic acid activates the pentose phosphate cycle and consumes nicotinamide adenine dinucleotide phosphate (NADPH). Lysates of EA.hy926 cells reduce α-lipoic acid using both NADPH and nicotinamide adenine dinucleotide (NADH) as electron donors, although NADPH-dependent reduction is about twice that due to NADH. NADPH-dependent α-lipoic acid reduction is mostly due to thioredoxin reductase. Pre-incubation of cells with α-lipoic acid increases their capacity to reduce extracellular ferricyanide, to recycle intracellular dehydroascorbic acid to ascorbate, to decrease reactive oxygen species generated by redox cycling of menadione, and to generate nitric oxide. These results show that α-lipoic acid enhances both the antioxidant defenses and the function of endothelial cells. © 2002 Elsevier Science Inc.

Keywords—Lipoic acid, Ascorbic acid, Menadione, Thioredoxin reductase, Oxidant stress, Nitric oxide, EA.hy926 cells, Free radicals

INTRODUCTION

α-Lipoic acid (LA) has been studied as an antioxidant with potential to improve endothelial function [1] and neuropathy in diabetes [2]. LA itself is not a strong antioxidant, but it becomes one when reduced to its dithiol form, dihydrolipoic acid (DHLA). This reduction is carried out within cells [3,4], although the DHLA generated does leak from the cells into the surrounding medium [4]. Because of this, the ability of cells to reduce LA to DHLA will have both intracellular and more distant antioxidant effects. For example, because DHLA can reduce both the ascorbate free radical and dehydroascorbic acid to ascorbate [5], it may also help to preserve this antioxidant vitamin either inside or outside cells, although this has not been demonstrated.

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Because the reduction potential of the LA/DHLA pair is lower than that of endogenous low molecular weight-reducing agents in cells, such as reduced glutathione (GSH) [6], the cellular reduction of LA to DHLA is likely to be enzyme-dependent. LA is not directly reduced by GSH [7], the most prominent intracellular thiol. Whether GSH can serve as a cofactor for LA reduction by such enzymes as glutaredoxin or protein disulfide isomerase is unknown. LA can be reduced by several cellular enzymes that require reduced pyridine nucleotides as co-factors. Lipoamide dehydrogenase, a component of pyruvate dehydrogenase and other keto-dehydrogenases, carries out nicotinamide adenine dinucleotide (NADH)-dependent reduction of LA in mitochondria [6]. LA can also be reduced in an nicotinamide adenine dinucleotide phosphate (NADPH)-dependent manner by glutathione reductase [3] and by thioredoxin reductase [8]. Measurement of rates of NADH- and NADPH-dependent LA reduction in cell lysates has been used to compare the relative contributions of these two mecha-

nisms to cellular LA reduction [9]. In neutrophils, NADH-dependent reduction is prominent [9], whereas in erythrocytes, which lack mitochondria, NADPH is the primary reducing co-factor for LA [3]. However, the relative contributions of glutathione reductase and thioredoxin reductase to NADPH-dependent LA reduction have not been assessed.

LA uptake and reduction has been studied mostly in cultured tumor cell lines and in erythrocytes. To provide more relevance for conditions characterized by oxidant stress, such as diabetes and atherosclerosis, we studied the extent and mechanism of uptake and reduction of LA by EA.hy926 cells. These cells are a permanent cell line developed from human umbilical vein endothelial cells that show the characteristics of endothelial cells, including factor VIII expression [10], oxidative modification of human low density lipoprotein [11], and calcium-dependent nitric oxide release [11]. Using a new method for assay of DHLA based on its derivatization in intact cells, we found that EA.hy926 cells rapidly take up and reduce LA to DHLA. Although most DHLA exits the cells, extracellular DHLA concentrations are maintained over 18–24 h in culture, suggesting a recycling mechanism. LA reduction by EA.hy926 cells is mostly dependent on thioredoxin reductase, although acute reduction of LA concentrations greater than 0.5 mM causes oxidant stress that depletes GSH and reduced pyridine nucleotides. Nonetheless, DHLA generated by low concentrations of LA enhances the ability of EA.hy926 cells to reduce extracellular ferricyanide and intracellular dehydroascorbic acid, protects them against oxidant stress due to intracellular recycling of menadione, and enhances their ability to generate nitric oxide.

EXPERIMENTAL PROCEDURES

Materials

EA.hy926 cells were a generous gift from Dr. Cora Edgell (University of North Carolina, Chapel Hill, NC, USA). The cells were cultured in Dulbecco's minimal essential medium (DMEM) that contained 20 mM D-glucose, 10% (v/v) fetal bovine serum, and HAT media supplement (Sigma/Aldrich Chemical Co., St. Louis, MO, USA). The cells were cultured to confluence for 18–24 h before use in an experiment. DL-LA, DL-DHLA, glutaredoxin-3, and mendione were from Sigma/Aldrich. Molecular Probes (Eugene, OR, USA) supplied the dihydrofluorescein diacetate and *N*-(1-pyrenyl)maleimide (pyrene maleimide). LA was dissolved in dimethylsulfoxide just before use, which resulted in a dimethylsulfoxide concentration of 0.4% (v/v) at an LA concentration of 1 mM. Radionuclides were purchased from New England Nuclear Life Science Products Inc (Boston, MA, USA).

Derivatization and assay of DHLA

DHLA was measured by a modification of the method of Winters et al. [12], which was used for assay of cellular GSH and thiol content. The assay is based on the reaction of pyrene maleimide with DHLA, followed by high performance liquid chromatography (HPLC) and fluorometric detection of the conjugate(s). The standard was prepared by mixing DHLA and a 6-fold molar excess of pyrene maleimide in acetonitrile, followed by dilution with a 25% volume of (N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES)-Krebs-Ringer buffer (HEPES-KRB: 20 mM HEPES, 128 mM NaCl, 5.2 mM KCl, 1 mM NaH_2PO_4, 1.4 mM $MgSO_4$, and 1.4 mM $CaCl_2$, pH 7.4). After incubation for 5 min at room temperature, the sample was acidified with an amount of glacial acetic acid equal to 1.25% of its volume (acidification is stated stabilize the derivative [12]). Chromatography of the derivatives was carried out at 23°C over a Bio-Rad (Hercules, CA, USA) C_8 clinical reverse-phase column using a mobile phase of 75% acetonitrile and 35% water containing 50 mM monochloroacetic acid at a flow rate of 1 ml/min. Peaks were detected with a Kratos Model 980 fluorometric detector, with filters chosen for an excitation wavelength of 330 nm and an emission wavelength of 380 nm. Under these conditions, mono-reacted lipoic acid eluted at 6–8 min, whereas the dimer eluted at 10–12 min. In most cell reactions, the monomer was the major peak quantified (>95%). Both peaks disappeared when a sample was treated with a 6-fold molar excess of *N*-ethylmaleimide in buffer before addition of pyrene maleimide. Assay sensitivity was 2 pmol per injection and the intra-assay variability was less than 5%. Derivatives of GSH and cysteine eluted in the void volume. Standards of the mono- and di-reacted products prepared as described above were quantified by ultraviolet (UV) spectroscopy using an extinction coefficient at for pyrene maleimide of 40 $mM^{-1}cm^{-1}$ at 338 nm. The molecular weight of the sodium salt of the dimer of pyrene maleimide with lipoic acid was confirmed by gas liquid chromatography and mass spectroscopy.

Assay of DHLA in cells and cell-conditioned buffer

For assay of DHLA in cell incubation buffer, after the indicated incubation, a 100 μl aliquot of the medium was mixed with 300 μl of a freshly prepared 2 mM solution of pyrene maleimide in acetonitrile. After 5 min, 4 μl of 50% (v/v) acetic acid in water was added, mixed by vortexing, and microfuged for 1 min. Aliquots of the supernatant were used for assay of DHLA. When DHLA was measured in cells from 6-well plates, medium was removed and the cells were rinsed twice in 4 ml of

ice-cold HEPES-KRB. Ice-cold buffer was used to prevent efflux of intracellular DHLA during the washes. The cell monolayer was treated with 0.1 ml of HEPES-KRB, followed by 0.3 ml of 2 mM pyrene maleimide in acetonitrile. After mixing for 5 min at room temperature, the cells were scraped from the plate with a plastic spatula, and the lysate was transferred to a microfuge tube. The lysate was microfuged for 1 min in the cold, and aliquots of the supernatant were taken for assay of DHLA. The intracellular concentration of DHLA was calculated based on the intracellular distribution space of 3-*O*-[^{14}C]methyl-D-glucose, corrected for trapped extracellular L-[$_3$H]glucose in the same sample, as recently described for bovine aortic endothelial cells [13]. For EA.hy926 cells, the intracellular water space in five determinations was 3.6 ± 1.2 μl/(mg protein).

Assay of radiolabeled glucose metabolism in EA.hy926 cells

Total glucose metabolism was assessed as the release of ^3H_2O from D-[2-^{3}H]glucose [14]. Pentose phosphate pathway activity was measured as the difference between release of $^{14}CO_2$ from D-[1-^{14}C]glucose and D-[6-^{14}C]glucose [15]. A 96-well plate containing cells was rinsed three times with 100 μl of DMEM, followed by addition of 100 μl of DMEM containing 5 mM D-glucose, the indicated concentration of LA, 0.1 μCi of D-[2-^{3}H]glucose, and 0.02 μCi of either D-[1-^{14}C]- or D-[6-^{14}C]glucose. The plate was covered with a flexible gas-tight mat and placed in a 37°C incubator. After 30 min, the medium was removed to a second 96-well plate and the remaining cells were fixed with 100 μl of 0.1 N NaOH and treated with 200 μl of scintillation fluid (Microscint-40; Packard Bioscience, Meriden, CT, USA) before radioactive counting in a 96-well Top-Counter (Packard Bioscience). A 96-hole gasket was placed over the plate containing the medium, followed by another 96-well plastic plate (Optiplate; Packard Bioscience) that contained 20 ul of 2N NaOH in each well. The plates were sealed to be airtight by placing a 20 lb lead brick on top. After 5–18 h at room temperature, the top plate was removed, 200 μl of scintillation fluid was added to each well of that plate, and the plate was covered. After mixing of the contents of the wells on a plate shaker, the radioactivity was counted. Radioactive counts per minute in low- and a high-energy windows were used in standard calculations to derive the contribution from either ^{3}H, or ^{14}C. It was found that ^3H_2O equilibrated freely from the gas phase into the two aqueous volumes of the system (120 μl total) with no detectable contamination from unreacted glucose in the well of the bottom plate. The $^{14}CO_2$ that was trapped in the NaOH droplet in the top plate was extracted with 95% recovery.

To test the effect of lipoic acid on glucose utilization, the indicated concentration of lipoic acid was added just before incubation with radionuclides. The activity of the pentose phosphate cycle was calculated by subtracting the nmol of $^{14}CO_2$ generated from D-[6-^{14}C]glucose (which reflects activity of the tricarboxylic acid cycle) from that generated from D-[1-^{14}C]glucose.

Assay of the pyridine nucleotide dependence of LA reduction in cell lysates

Dependence of LA reduction on NADPH or NADH in cell lysates was measured by a modification of the method of Haramaki et al. [9]. EA.hy926 cells in 10 cm plates were rinsed three times in 2 ml of HEPES-KRB and scraped from the plate in 4 ml of ice-cold HEPES-KRB. The suspension was subjected to two freeze–thaw cycles using dry ice-acetone. The lysate was centrifuged for 4 min at 400 × *g* to remove unbroken cells, and the resulting supernatant homogenized with a Teflon-glass homogenizer. Rates of NADH or NADPH reduction in lysate were measured in a Beckman DU-640 spectrophotometer at 340 nm, taking readings every 12 s for 3 min at 37°C. Incubations contained lysate, 80 μM NADH or NADPH, 1 mM LA, and other agents as noted. The rate of LA reduction was calculated as the rate of decrease in nucleotide absorbance, corrected for the rate in a blank that contained buffer, lysate and nucleotide alone. Glutathione reductase activity was measured in a sample that contained lysate, 0.8 mM glutathione disulfide (GSSG), NADPH, but no LA. NADPH-dependent LA reduction was measured in the presence of lysate, NADPH, LA, and 5 μM thioredoxin. A parallel reaction was measured with the same additions as well as 10 μM aurothioglucose to inhibit thioredoxin reductase [16]. Thioredoxin reductase activity was taken as aurothioglucose-sensitive NADPH reduction. NADH-dependent reduction was measured in the presence of lysate, NADH, and LA.

Intracellular oxidant stress measured by oxidation of dihydrofluorescein

Intracellular reactive oxygen species were measured as oxidation of intracellular dihydrofluorescein [17]. Dihydrofluorescein diacetate is taken up by the cells and de-esterified. Removal of the acetate groups traps dihydrofluorescein within the cells, where it can be oxidized to fluorescein with a marked increase in fluorescence. Although dichlorofluorescein is most frequently used in this assay [17], our results agree with those of Hempel et al. [18] that dihydrofluorescein provides better sensitivity. EA.hy926 cells were grown to confluence in 96-well plates suitable for fluorescence measurements (Corning

Costar Model 3904 assay plate; Corning, NY, USA). The cells were rinsed twice in HEPES-KRB to remove culture medium, and incubated in 0.2 ml of HEPES-KRB that contained 5 mM glucose, the indicated concentration of LA, and 20 μM dihydrofluorescein diacetate. The latter was prepared to a 10 mM concentration in dimethylsulfoxide and stored at −20°C until dilution for use in HEPES-KRB. The cells were incubated for 30 min at 37°C, rinsed three times with 0.2 ml of HEPES-KRB, and then incubated further in 0.2 ml of HEPES-KRB that contained 5 mM glucose and 80 μM menadione (vitamin K_3). The plate was loaded into a fluorescence microtiter plate reader (Fluostar Galaxy; BMG Labtechnologies, Cork, Ireland) and incubated at 37°C for 36 min with measurement of the fluorescence in each well every 4 min. The excitation wavelength was 480 nm, and the emission wavelength was 520 nm. The fluorescence readings for each well were normalized to the initial reading at time zero. It was found that the fluorescence detected by the plate reader was due to fluorescein that had leaked out of the cells into the incubation medium during the 30 min incubation. However, menadione did not directly oxidize dihydrofluorescein, so that changes in extracellular fluorescence were considered to reflect the effects of intracellular oxidation of dihydrofluorescein. Further, a 1000-fold molar excess of DHLA did not reduce fluorescein.

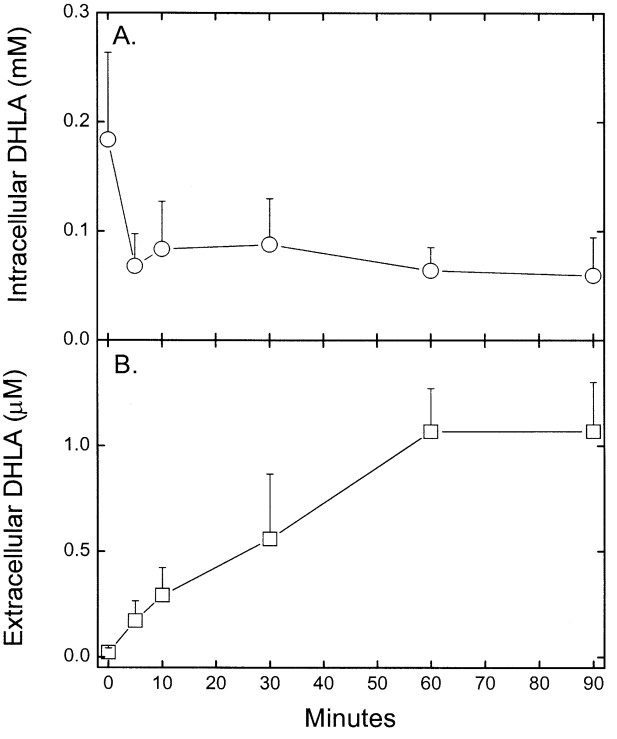

Fig. 1. Time course of LA uptake and reduction. EA.hy926 cells that had been rinsed free of medium were incubated in 2 ml of HEPES-KRB buffer that contained 5 mM D-glucose and 0.5 mM LA for the indicated times before assay of DHLA in the cells (A) and medium (B) as described in Experimental Procedures. Results are shown from five experiments as mean ± SE.

Other assays

Ferricyanide reduction was measured as the amount of ferrocyanide generated by cells in an aliquot of incubation medium after a 30 min incubation at 37°C. Ferrocyanide was measured by the method of Avron and Shavit [19], using *ortho*-phenanthroline to detect ferrocyanide, and correcting for absorption in buffer from cells that were incubated without ferricyanide. Intracellular concentrations of GSH were measured as previously described [20] using the method of Hissin and Hilf [21]. Pyridine nucleotide concentrations were measured by the recycling method of Zerez et al. [22] in 1 ml lysates from cells cultured in 6-well plates. Intracellular ascorbate was measured as previously described in cultured cells [23] using HPLC with electrochemical detection [24], except that tetrapentylammonium bromide was used as an ion pair reagent. Intracellular concentrations of GSH, pyridine nucleotides, and ascorbate were expressed relative to the intracellular distribution space of 3-*O*-[^{14}C]methylglucose, as noted for DHLA. It should be noted that these intracellular substances may not have the same intracellular distribution spaces as 3-*O*-methylglucose, so their intracellular concentrations can only be considered approximations. Glutaredoxin activity was measured either directly as the appearance of DHLA from 2 mM LA in the presence of 2 mM GSH, or in a coupled assay using an excess of glutathione reductase (0.42 U/ml), 0.2 mM NADPH, 0.5 mM GSH, and LA in 100 mM sodium phosphate buffer, pH 7.5 [25]. Nitric oxide synthesis was measured in intact cells as the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, corrected for L-[^{3}H]arginine uptake, as described previously [13]. Protein was determined using the Bradford method (Bio-Rad), according to the manufacturer's instructions.

RESULTS

At a concentration of 0.5 mM, lipoic acid was rapidly taken up by EA.hy926 cells and reduced to DHLA (Fig. 1). Once generated, DHLA effluxed into the incubation buffer so rapidly that the highest intracellular concentration was reached at the first time point that could be taken, which was about 1 min after adding LA (Fig. 1A). This reflects reduction of LA that had entered cells before extracellular LA could be removed by washes. After the initial time point, the intracellular concentration of DHLA remained relatively stable at just under 100 μM during a subsequent 90 min incubation. Extracellular DHLA increased with time to a maximal value of 1 μM

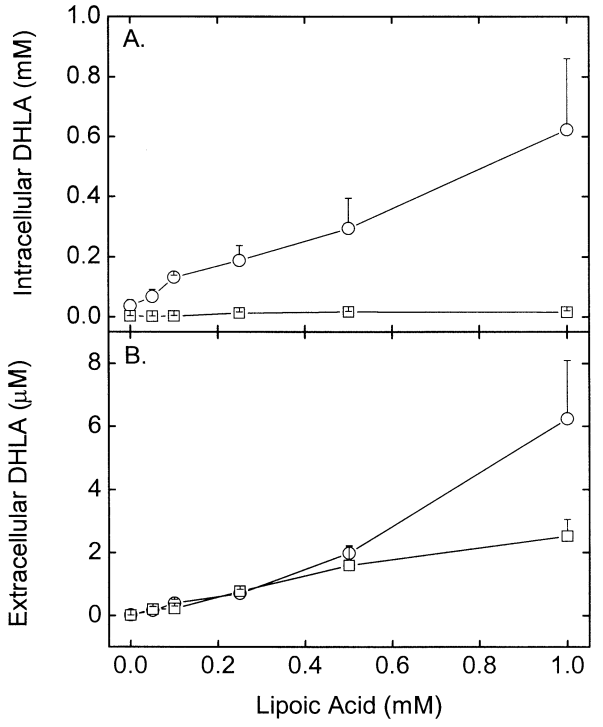

Fig. 2. Concentration dose-response of LA uptake and reduction. In acute uptake studies shown by the circles, rinsed cells were incubated at 37°C in 2 ml of HEPES-KRB buffer that contained 5 mM D-glucose and the indicated initial concentration of LA. After 30 min, DHLA was assayed in cells (A) and medium (B). In overnight loading studies shown by the squares, cells were incubated in culture for 16–18 h with the indicated initial concentration of LA, followed by assay of intracellular (A) and extracellular (B) DHLA. Results are shown as mean ± SE from three experiments for the 30 min incubation, and from five experiments for the overnight incubation.

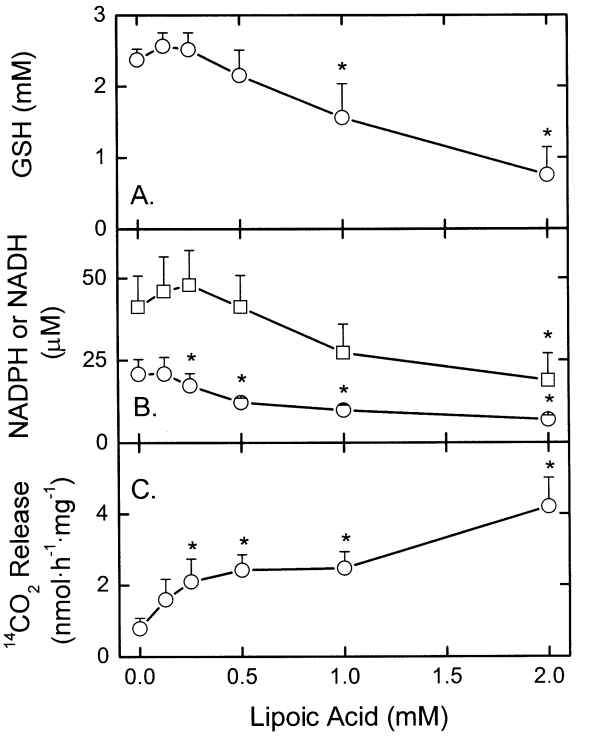

Fig. 3. Effects of LA on cellular GSH, NADH, NADPH, and pentose phosphate cycle activity. For data shown in (A) and (B), EA.hy926 cells were incubated at 37°C in HEPES-KRB that contained 5 mM D-glucose and the indicated concentration of LA. After 15 min, the buffer was removed and the cells rinsed twice in 2 ml of HEPES-KRB and taken for assay of GSH (A, $n = 4$), NADPH (B, circles, $n = 8$), and NADH (B, squares, $n = 5$). Assay of glucose carbon-1 oxidation to CO_2 (C) was carried out over 30 min at 37°C as described under Experimental Procedures. An asterisk (*) indicates $p < .05$ compared to a control sample not treated with LA (two-way analysis of variance and post-hoc testing by Dunnett's test).

at about 60 min (Fig. 1B). Although intracellular DHLA concentrations almost reached 1 mM, the great majority (97–98%) of the total DHLA generated was released from the cells. When EA.hy926 cells were incubated with increasing concentrations of LA for 30 min, the DHLA concentrations within the cells rose in proportion to the extracellular DHLA concentration (Fig. 2A, circles). Extracellular DHLA increased in proportion to the initial loading concentration of LA, reaching concentrations as high as 6 μM in the medium at the end of the incubation (Fig. 2B, circles). In these experiments, the total DHLA reduced by the cells across the range of LA concentrations added was 4 ± 1.6% of the LA added.

EA.hy926 cells were much less able to maintain intracellular DHLA when incubated overnight in culture than following a 30 min incubation (Fig. 2A, squares). Intracellular DHLA was undetectable at initial LA concentrations below 0.2 mM, but rose significantly to about 16 μM at the two highest LA concentrations ($p < 0.05$ for both 0.5 and 1.0 mM LA, compared to no LA). Extracellular DHLA concentrations rose in proportion to the loading concentration of LA, and were for the most part similar to those observed during a short uptake period (Fig. 2B, squares). Although most of the DHLA is found in the medium after short or overnight incubations, the cells are capable of sustained maintenance of extracellular DHLA.

To determine the source of reducing equivalents for acute reduction of LA, we studied the effects of LA loading on concentrations of potential intracellular reducing agents. As shown in Fig. 3A, intracellular GSH was unaffected by a 15 min incubation with concentrations of LA less than 0.5 mM. Above that LA concentration, intracellular GSH concentrations decreased, although to only about 30% of control levels. In an attempt to determine whether direct or enzyme-dependent LA reduction contributed to the decrease in GSH, lysates of EA.hy926 cells that had been prepared as described under Experimental Procedures were incubated for 15 min at 37°C with 0.5 mM GSH and 100 μM LA. No DHLA generation was evident under these conditions (results not shown). Commercial glutaredoxin-3 did en-

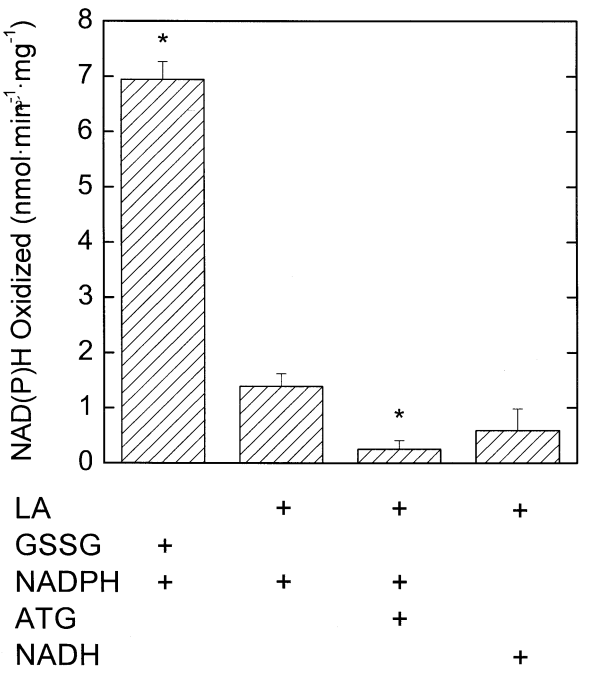

Fig. 4. Pyridine nucleotide oxidation by EA.hy926 cell lysates. Lysates prepared from EA.hy926 cells were assayed for oxidation of pyridine nucleotide oxidation as described under Experimental Procedures for each of the treatments noted (ATG = aurothioglucose). Results are shown from five experiments, with an asterisk (*) indicating $p < .05$ compared to LA plus NADPH by one-way analysis of variance, and post-hoc testing by Dunnett's test.

hance GSH-dependent reduction of 100 μM LA under these conditions, but with a k_{cat} of only 0.64 $\pm$ 0.08 min^{-1}.

Incubation of EA.hy926 cells with increasing amounts of LA caused a progressive decrease in intracellular NADPH to concentrations that were about one-third of control values at 2 mM LA (Fig. 3B, circles). NADH also decreased in response to LA, although differences only became significant at 2 mM due to variability between different cell cultures (Fig. 3B, squares). LA uptake and reduction was associated with activation of the pentose phosphate cycle in response to LA (Fig. 3C). There was no change in the rate of [2-^{3}H]glucose utilization over this range of LA concentrations (results not shown). Together, these results show that LA reduction is supported to a small extent by electrons derived from glucose metabolism in the pentose phosphate cycle, and that concentrations of LA greater than 0.5 mM deplete both GSH and reduced pyridine nucleotides.

To directly compare rates of LA reduction by NADPH- and NADH-dependent mechanisms, cell lysates were prepared to contain cell membranes including mitochondria and incubated as shown in Fig. 4. NADPH reduction was more than 5-fold greater with GSSG as substrate than with LA, showing that glutathione reductase was active in this preparation. Most NADPH-dependent LA reduction was due to thioredoxin reductase rather than to glutathione reductase, because it was inhibited 80% by 10 μM aurothioglucose (Fig. 4). The latter specifically inhibits thioredoxin reductase at this concentration, and does not inhibit glutathione reductase [16]. NADPH-dependent LA reduction due to thioredoxin reductase was 1.15 nmol $\times$ min^{-1} $\times$ (mg protein)$^{-1}$, which was about twice that observed for NADH-dependent LA reduction 0.59 nmol $\times$ min^{-1} $\times$ (mg protein)$^{-1}$. These results suggest that in EA.hy926 cells a relatively high concentration of LA is reduced primarily, although not exclusively by NADPH through the thioredoxin reductase system.

To estimate the capacity of EA.hy926 cells to recycle LA, cells were loaded with LA or dehydroascorbic acid and their ability to reduce ferricyanide was compared. Ferricyanide, which itself does not enter cells [26], will oxidize DHLA or ascorbate directly, and will oxidize intracellular ascorbate via a transplasma membrane oxidoreductase [27]. The extent to which cells can reduce ferricyanide provides an estimate of their ability to regenerate DHA [20,28], and this should also apply to DHLA. Cells were treated for 15 min with increasing concentrations of LA or dehydroascorbic acid and incubated for an additional 30 min with 1 mM ferricyanide. EA.hy926 cells reduced ferricyanide in the absence of LA, and this reduction was progressively increased with increasing initial concentrations of either LA or dehydroascorbic acid. However, the rate of ferricyanide reduction in response to dehydroascorbic acid was greater than that due to LA, especially at low dehydroascorbic acid concentrations (Fig. 5).

Because DHLA can reduce dehydroascorbic acid to ascorbate [5], we next determined whether loading EA.hy926 cells with DHLA affects their ability to generate ascorbate from its two-electron-oxidized form, dehydroascorbic acid. Pre-incubation of cells with increasing concentrations of LA progressively enhanced the ability of the cells to reduce intracellular dehydroascorbic acid to ascorbate (Fig. 6). GSH concentrations were lowered slightly by these treatments, but less than by a single exposure to LA over a shorter period of time (Fig. 3A). These results show that LA enhances the ability of EA.hy926 cells to recycle ascorbate with minimal oxidant stress to the cells.

We also tested whether DHLA inside cells could decrease intracellular oxidant stress due to redox cycling of menadione. As shown in Fig. 7, incubation of cells with menadione alone caused progressive oxidation of dihydrofluorescein, which then leaked out of the cells and was detected in the incubation medium (Fig. 7, circles). Pre-incubation of the cells for 30 min with LA caused a dose-dependent inhibition of this oxidation, with the largest relative effect observed at the lowest LA

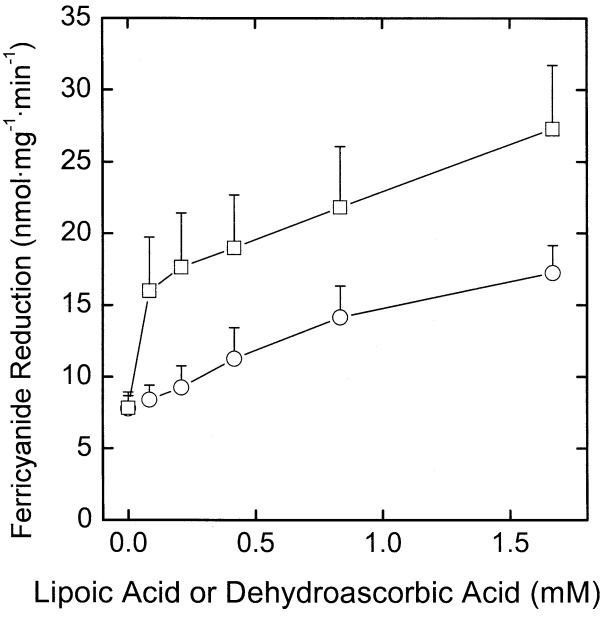

Fig. 5. Ferricyanide reduction by EA.hy926 cells. Rinsed cells were incubated at 37°C in HEPES-KRB that contained 5 mM D-glucose and the indicated concentration of LA (circles) or dehydroascorbic acid (squares). After 15 min, the cells were rinsed twice in 2 ml of warm HEPES-KRB and incubated for an additional 30 min in HEPES-KRB that contained 5 mM D-glucose and 1 mM potassium ferricyanide. Aliquots of the medium were removed for assay of ferrocyanide as described in Experimental Procedures. Results are shown for seven experiments.

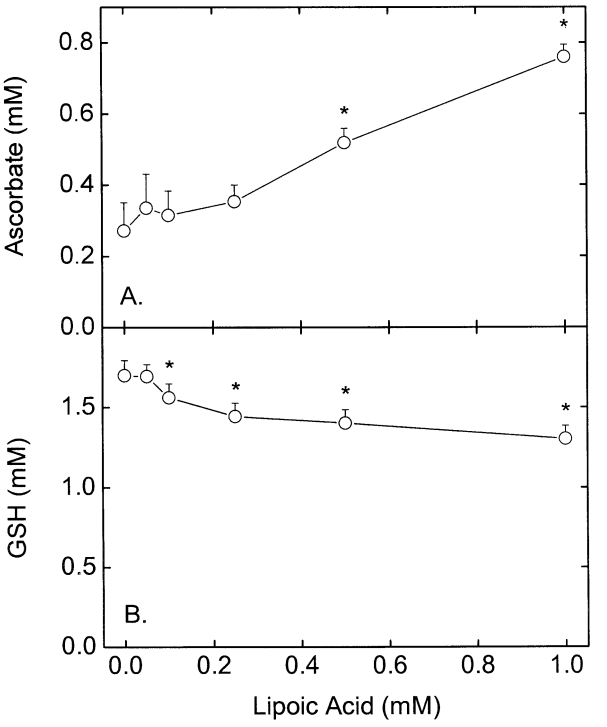

Fig. 6. Dehydroascorbic acid reduction by LA in EA.hy926 cells. EA.hy926 cells were incubated for 15 min at 37°C with the HEPES-KRB containing 5 mM D-glucose and the indicated concentration of LA. Freshly prepared dehydroascorbic acid was added to an initial concentration of 0.25 mM, and the cells were incubated for an additional 15 min. The cells were rinsed three times in 2 ml of HEPES-KRB and taken for assay of intracellular ascorbate (A) and GSH (B). Results are shown from five experiments, with an asterisk indicating $p < .05$ compared to cells not treated with LA (two-way analysis of variance and post-hoc testing by Dunnett's test).

concentrations used. The decrease in the rate of oxidation of dihydrofluorescein followed a monoexponential decay (inset to Fig. 7), such that the t½ occurred at an initial extracellular LA concentration of 70 μM. Based on the measurements shown in Fig. 2A under the same conditions, this corresponds to an intracellular DHLA concentration of about 50 μM. Similar findings were obtained in two additional experiments. These results show that a short-term incubation of EA cells with LA decreases endogenous oxidant stress due to redox cycling by menadione.

To assess effects of LA on endothelial cell function, we measured nitric oxide synthesis in intact cells as the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline. Incubation of EA.hy926 cells with LA for 30 min inhibited basal and A23187-stimulated L-[^{3}H]arginine uptake and conversion to L-[^{3}H]citrulline by 80%, with a half-maximal effect at 150–200 μM LA (results not shown). However, as shown in Fig. 8, a 16–18 h incubation of cells with increasing concentrations of LA enhanced both basal and A23187-stimulated nitric oxide synthesis. Basal activity was stimulated by about 30% by LA treatment. A brief treatment of the cells with A23187 to increase intracellular calcium doubled the activity of endothelial nitric oxide synthase. LA pretreatment enhanced A23187-stimulated activity by 50–75% in a concentration-dependent manner. For both control and A23187-treated cells, significant effects were seen at LA concentrations as low as 100 μM. Uptake of L-[^{3}H]arginine was also measured in this assay, and was unaffected by the 16–18 hr treatment with LA (results not shown).

DISCUSSION

To assess cellular effects of LA, it is necessary to have a sensitive and specific assay for its reduced form, DHLA. The latter is rapidly generated in cells and likely mediates the antioxidant effects of the agent. Packer's group [29,30] have developed direct HPLC assays for LA and DHLA using electrochemical detection. The current assay differs in that it is based on reaction of DHLA with an excess of pyrene maleimide in intact cells or medium. Separation of the reaction product from that of conjugates of pyrene maleimide with cellular thiols such as GSH is carried out by HPLC, followed by detection of the intensely fluorescent conjugate. This

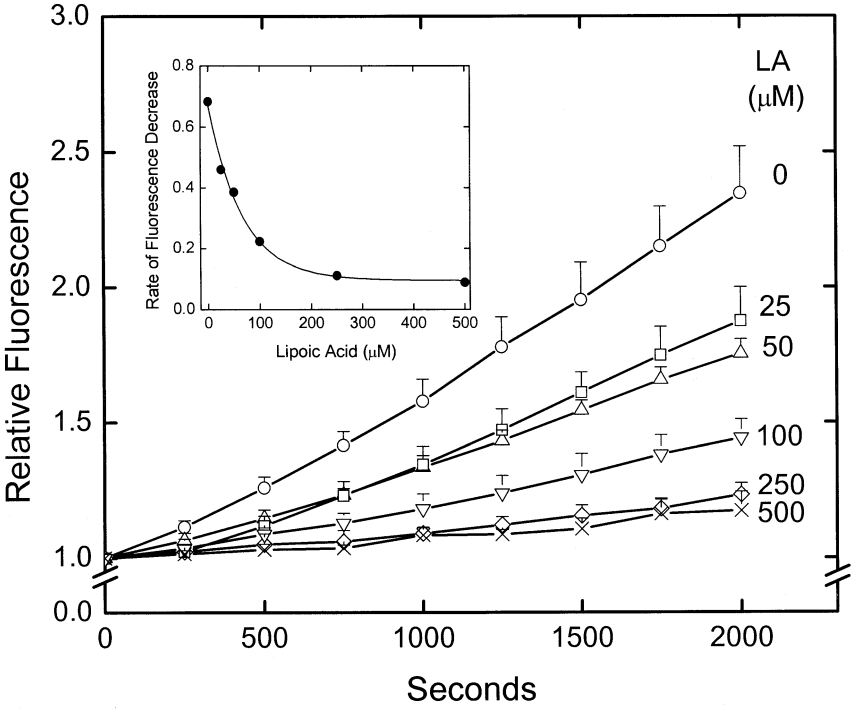

Fig. 7. Time-dependent protection against dihydrofluorescein oxidation due to menadione in EA.hy926 cells. Cells that had been pre-treated with the indicated concentration of LA and 20 μM dihydrofluorescein for 30 min at 37°C were washed and incubated as described in Experimental Procedures with 80 μM menadione during measurement of fluorescence. The inset shows a monoexponential fit to the slopes of each line in the main figure.

assay has a similar sensitivity for DHLA to the method using direct electrocoulometric detection of DHLA (2-5 pmol/injection) [30] and is 8–10-fold more sensitive than the method using a custom mercury/gold electrode [29]. Further, by derivatizing DHLA in intact cells or medium, the current assay minimizes the risk of DHLA oxidation during cell lysis and sample preparation. Negative aspects of the current assay are that it does not allow detection of LA, and that it is necessary to synthesize standards of the DHLA conjugate with pyrene maleimide.

Using this assay, we find that rates of LA uptake, reduction, and release by human-derived endothelial cells are similar to those observed in human erythrocytes [3], cultured tumor cell lines [4], and fibroblasts [4]. EA.hy926 cells rapidly take up and reduce LA to DHLA, which is mostly released into the incubation medium (Fig. 1). The LA-reducing capacity of EA.hy926 cells is such that they can sustain DHLA both inside and outside of cells in overnight culture, although intracellular concentrations are markedly lower than those present after a 30 min incubation (Fig. 2). In contrast to endogenous water-soluble low molecular weight antioxidants, such as GSH and ascorbate, which are concentrated against a gradient within cells, LA may have prominent extracellular effects. For example, LA treatment of cells for several hours in culture increases intracellular GSH concentrations [31,32]. This effect is due to reduction of cystine to cysteine in the culture medium by DHLA released from the cells, followed by cellular uptake of cysteine and incorporation into GSH [32]. The tendency for cells to release rather than retain DHLA may provide a unique mechanism for its action.

In contrast to the ability of LA to increase intracellular GSH in overnight cell culture noted above, acute incubation of intact EA.hy926 cells at 37°C for 15 min with increasing concentrations of LA depletes GSH at LA concentrations of 1 mM and greater (Fig. 3A). GSH depletion was not explained by direct oxidation by LA, or to loss of GSH from reduction due to glutaredoxin. Although commercial glutaredoxin-3 enhanced LA reduction to DHLA, the k_{cat} of this activity for LA (0.6 min^{-1}) was less than 5% of that of lipoamide dehydrogenase (k_{cat} = 20.3 min^{-1}) and less than 0.3% that of thioredoxin reductase (k_{cat} = 297 min^{-1}) [8]. The fall in cellular GSH content, along with oxidation of both NADPH and NADH (Fig. 3B), suggests that the reducing capacity of the cells is taxed at high LA concentrations, such that GSH is oxidized in response to increased oxidant stress within the cells. Although the use of high LA concentrations helps to define the mechanism of its reduction, it is unlikely that LA concentrations beyond

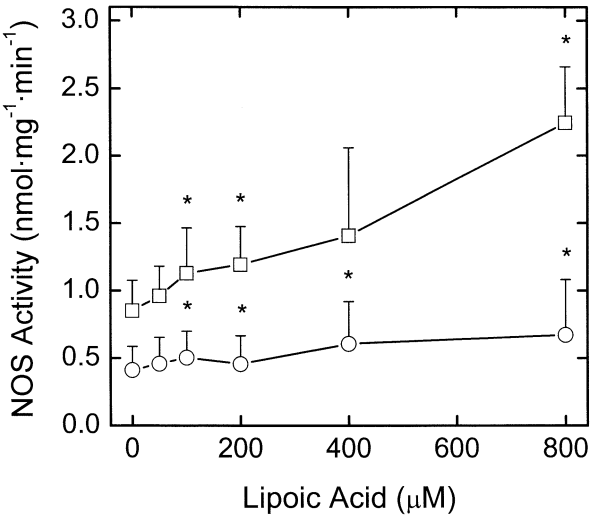

Fig. 8. Stimulation of nitric oxide synthase activity in EA.hy926 cells by LA. Cells in 6-well plates were incubated for 16–18 h in overnight culture in HEPES-KRB containing 5 mM D-glucose and the indicated concentration of LA. Then the cells were rinsed twice in HEPES-KRB and incubated for 3 min in HEPES-KRB containing 5 mM D-glucose at 37°C in the absence (circles) or presence of 2.5 μM A32187 (squares) before addition of 0.5 μCi/ml of L-[^{3}H]arginine (9.4 μM). After incubation for an additional 15 min at 37°C, the cells were assayed for L-[^{3}H]arginine uptake and conversion to L-[^{3}H]citrulline exactly as previously described [13]. Results are shown from six experiments as mean ± SE, with an asterisk indicating $p < .05$ compared to a sample without added LA by two-way analysis of variance and Dunnett's test.

the low micromolar range would ever be achieved in vivo following administration to humans.

LA is reduced to DHLA within cells by several enzymes. Lipoamide dehydrogenase, a mitochondrial enzyme, reduces LA through an NADH-dependent mechanism [33]. Glutathione reductase reduces LA using NADPH as the electron donor, but with only about 11% the efficiency of its natural substrate, GSSG [3]. Thioredoxin reductase is also capable of NADPH-dependent LA reduction, a reaction that is 15-fold more efficient than that with lipoamide dehydrogenase [8]. In lysates of EA.hy926 cells and using an enantiomeric mixture of LA as substrate, rates of NADPH-dependent LA reduction due to thioredoxin reductase are about twice those seen with NADH (Fig. 4). Although glutathione reductase has been implicated in reduction of LA by human erythrocytes [3], those results could just as well have been due to thioredoxin reductase, which is also present in erythrocytes [34]. In EA.hy926 cells, despite the presence of glutathione reductase, NADPH-dependent LA reduction that can be attributed to glutathione reductase is less than 25% of that due to thioredoxin reductase (Fig. 4). The finding that thioredoxin reductase accounts for most LA reduction by EA.hy926 cells contrasts with the results found in rat cardiac muscle, liver, and brain [9], which use mitochondrial lipoamide dehydrogenase to reduce LA.

DHLA generated from LA by EA.hy926 cells reduces extracellular ferricyanide (Fig. 5) and thus ameliorates the oxidant stress due to this agent. Given the observation that most DHLA rapidly effluxes from cells (Figs. 1 and 2), ferricyanide was probably reduced directly by extracellular DHLA. In contrast, intracellular ascorbate reduces extracellular ferricyanide via a transplasma membrane oxidoreductase known to be present in endothelial cells [20,28]. The finding that ferricyanide reduction is greater with dehydroascorbic acid than with LA across the same concentration range for each agent (Fig. 5) suggests either that dehydroascorbic acid reduction is more efficient than that of LA, or that ascorbate oxidation products (the ascorbate free radical and dehydroascorbic acid) are retained in cells and thus have better access to reducing systems than extracellular LA. On the other hand, DHLA generated by the cells enhances their ability to reduce dehydroascorbic acid to ascorbate (Fig. 6). Because ascorbate was measured within the cells, and because ascorbate enters endothelial cells slowly compared to rates of uptake and reduction of dehydroascorbic acid [35], intracellular rather than extracellular DHLA is responsible for reduction of dehydroascorbic acid. Whereas most DHLA leaves the cells, that remaining within the cells is still quite capable of increasing ascorbate recycling, which in turn will enhance the antioxidant capacity of the cells.

An increase in cellular antioxidant capacity due to LA was confirmed by the finding that pre-incubation of EA.hy926 cells with LA caused a dose-dependent inhibition of dihydrofluorescein oxidation due to menadione (Fig. 7). Menadione has previously been shown to increase H_2O_2 generation in cultured bovine aortic endothelial cells [36]. Most of this H_2O_2 is probably generated in mitochondria, which are known to redox cycle menadione [37,38]. This suggests that DHLA may help to decrease intracellular oxidant stress generated during normal mitochondrial respiration, because as much as 1–2% of the oxygen metabolized by mitochondria is released as superoxide and ultimately as H_2O_2 after the action of superoxide dismutase [39].

Our finding that overnight incubation of EA.hy926 cells with LA enhances basal and calcium-stimulated nitric oxide synthase activity in intact cells (Fig. 8) suggests that LA has beneficial effects on endothelial cell function. In contrast to the results seen in overnight culture, acute incubation of endothelial cells with LA inhibits L-[^{3}H]arginine uptake and subsequent conversion to L-[^{3}H]citrulline. This may explain the results of previous studies showing that both the endothelial [40] and inducible forms [41,42] of nitric oxide synthase are inhibited by acute or short-term incubation of cells with

LA. Ascorbate has also been shown to stimulate endothelial cell nitric oxide synthase activity, an effect that also required prolonged incubation with ascorbate in culture [43]. The effect of ascorbate appears to be due to sparing of tetrahydrobiopterin, a required co-factor of endothelial nitric oxide synthase [44,45]. Whether the same mechanism accounts for the effects of LA will require further study. Whatever the mechanism, our results provide confirmation at the cell level of the recent observation that LA administration can preserve nitric oxide-dependent vascular relaxation in human diabetes [1].

In conclusion, using an assay for DHLA that minimizes oxidative loss of DHLA during cell extraction and sample work-up, we find that cultured endothelial cells readily take up and reduce LA to DHLA, and that the mechanism of acute NADPH-dependent reduction involves thioredoxin reductase rather than glutathione reductase. The DHLA generated from LA reduction by EA.hy926 cells enhances their ability to reduce extracellular ferricyanide, to recycle DHA to ascorbate, and to consume reactive oxygen species generated by menadione. Such antioxidant effects of DHLA may help to explain its ability to enhance nitric oxide generation by endothelial cells.

Acknowledgements — This work was supported by National Institutes of Health (NIH) grant AG 16236 and by the Vanderbilt Summer Diabetes Program (NIH grant DK 07383).

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ABBREVIATIONS

DHLA—dihydrolipoic acid
DMEM—Dulbecco's minimal essential medium
HEPES-KRB—HEPES-Krebs-Ringer buffer
HPLC—high-performance liquid chromatography
LA—α-lipoic acid
Pyrene maleimide—*N*-(1-pyrenyl)maleimide