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Modulation of Cellular Reducing Equivalent Homeostasis by α-Lipoic Acid

MECHANISMS AND IMPLICATIONS FOR DIABETES AND ISCHEMIC INJURY

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ABSTRACT. The therapeutic potential of α-lipoic acid (thioctic acid) was evaluated with respect to its influence on cellular reducing equivalent homeostasis. The requirement of NADH and NADPH as cofactors in the cellular reduction of α-lipoic acid to dihydrolipoate has been reported in various cells and tissues. However, there is no direct evidence describing the influence of such reduction of α-lipoate on the levels of cellular reducing equivalents and homeostasis of the NAD(P)H/NAD(P) ratio. Treatment of the human Wurzburg T-cell line with 0.5 mM α-lipoate for 24 hr resulted in a 30% decrease in cellular NADH levels. α-Lipoate treatment also decreased cellular NADPH, but this effect was relatively less and slower compared with that of NADH. A concentration-dependent increase in glucose uptake was observed in Wurzburg cells treated with α-lipoate. Parallel decreases (30%) in cellular NADH/NAD^+ and in lactate/pyruvate ratios were observed in α-lipoate-treated cells. Such a decrease in the NADH/NAD^+ ratio following treatment with α-lipoate may have direct implications in diabetes, ischemia–reperfusion injury, and other pathologies where reductive (high NADH/NAD^+ ratio) and oxidant (excess reactive oxygen species) imbalances are considered as major factors contributing to metabolic disorders. Under conditions of reductive stress, α-lipoate decreases high NADH levels in the cell by utilizing it as a co-factor for its own reduction process, whereas in oxidative stress both α-lipoate and its reduced form, dihydrolipoate, may protect by direct scavenging of free radicals and recycling other antioxidants from their oxidized forms. *Copyright © 1997 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;3: 393–399, 1997.

KEY WORDS. thioctic acid; reductive stress; oxidative stress; hypoxia; ischemia

Cellular redox imbalances in hyperglycemic (e.g. diabetic) and hypoxic (e.g. ischemic) tissue are one of the fundamental factors that contribute to the complications of these pathological disorders. In hypoxic tissues, impaired mitochondrial oxidation of NADH to NAD^+ because of decreased pO_2 results in an increased intracellular NADH/NAD^+ ratio [1]. In hyperglycemia, the rate of oxidation of glucose to sorbitol by aldose reductase activity increases. Subsequently, sorbitol dehydrogenase activity increases the intracellular NADH/NAD^+ ratio by oxidation of sorbitol to fructose, resulting in a situation commonly referred to as pseudohypoxia [2]. An elevated cellular NADH/NAD^+ ratio causes "reductive stress." Several similarities have been observed between functional abnormalities associated with high NADH/NAD^+ under conditions of diabetes and ischemia [2]. A high intracellular NADH/NAD^+ ratio inhibits several major metabolic pathways such as glycolysis and fatty acid oxidation [2], and exacerbates the intracellular formation of additional ROS[‖] that may cause cell injury [3]. Oxidation of NADH to NAD^+ by the mitochondrial respiratory chain is associated with the one-electron reduction of O_2 generating $O_2^{\cdot-}$. Under "reductive stress" conditions, elevated cytosolic NADH equivalents are transported rapidly into mitochondria where auto-oxidation of reduced electron transport components (such as ubiquinol and/or flavoproteins) may further enhance $O_2^{\cdot-}$ formation [2]. Furthermore, high NADH/NAD^+ may result in the release of cellular stores of iron (Fe^{3+}) bound to ferritin, resulting in free reduced Fe^{2+} that may catalyze Fenton reactions leading to the production of hydroxyl radicals [4].

Recent studies have highlighted considerable interest in the therapeutic value of the metabolic antioxidant α-lipoic acid or thioctic acid (1,2-dithiolane-3-pentanoic acid), especially with regard to pathologies that have redox imbalances [5, 6]. Beneficial effects of α-lipoic acid administration have been reported in diabetic complications and ischemia–reperfusion injury [7–11]. α-Lipoic acid has been used

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‖ *Abbreviations:* ROS, reactive oxygen species; $O_2^{\cdot-}$, superoxide anion radical; DHLA, 6,8-dithiooctanoic acid (dihydrolipoate); FBS, fetal bovine serum; GSH, glutathione; and GSSG, glutaehione disulfide.

for a long time in Germany to treat complications associated with diabetes [7]. Recently, α-lipoic acid has also been introduced in the United States as a metabolic antioxidant. Apart from the essential role of α-lipoic acid as a co-factor in oxidative metabolism, much of the current interest has been centered on the antioxidant properties of exogenously supplied α-lipoic acid, which is a free and non-protein bound compound.

Under physiological conditions, α-lipoic acid is found as lipoamide in five proteins in eukaryotes where it is covalently bound to a lysyl residue. Four of these proteins are found in the α-keto acid dehydrogenase complex, the pyruvate dehydrogenase complex, and the branched chain keto acid complex. The fifth lipoamide moiety is in the glycine cleavage system [12]. Exogenously supplied α-lipoic acid is taken up readily by a variety of cells and tissues where it is reduced rapidly by NADH- or NADPH-dependent enzymes to dihydrolipoate (DHLA) [13, 14]. Reduction of α-lipoic acid to DHLA has been also observed *in vivo* [15]. A major portion of DHLA is released promptly by the cells to the extracellular medium. The E3 enzyme, dihydrolipoamide dehydrogenase (EC 1.8.1.4), of the mitochondrial α-keto acid dehydrogenase complex and the pyruvate dehydrogenase complex, is capable of reducing α-lipoic acid to DHLA. Dihydrolipoamide dehydrogenase shows a marked preference for the natural, *R*-enantiomer of α-lipoic acid, compared with the *S*-enantiomer [16]. In addition, NADPH-dependent reduction of α-lipoic acid to DHLA has been observed in cells and tissues. However, except in some cell types such as human erythrocytes that lack mitochondria, it is reported that NADH-dependent reduction of α-lipoic acid is the major pathway and that the NADPH-dependent pathway of reduction is relatively minor [12, 14]. Although it is evident that NADH and NADPH are essential cofactors required for the biological reduction of α-lipoic acid to DHLA, there is no direct evidence describing the influence of exogenously supplied α-lipoic acid on cellular homeostasis of the NAD(P)H to NAD(P) ratio. We investigated α-lipoic acid with respect to its reduction to DHLA and the effects of this process on the homeostasis of cellular reducing equivalents with the aim of evaluating the possible therapeutic potentials of α-lipoic acid in reductive and oxidative stress-dependent pathologies.

MATERIALS AND METHODS

Cell and Cell Culture

Wurzburg T-cells, a subclone of human Jurkat T-cells (American Type Culture Collection, ATCC, Bethesda, MD), developed by Dr. Patrick Baeuerle (Freiburg, Germany) were a gift of Dr. Leonard Herzenberg of Stanford University [17]. Cells were grown in RPMI 1640 medium supplemented with 10% FBS, 1% (w/v) penicillin/streptomycin, 110 mg/L sodium pyruvate and 2 mM L-glutamine (University of California, San Francisco) in humidified air containing 5% CO_2. For experiments, cells (10^6 cells/mL) were suspended in the above-mentioned medium and treated with different concentrations of the *R*-enantiomer of α-lipoic acid for time periods as indicated in the figure legends. Cells were maintained in a culture incubator with humidified air containing 5% CO_2 at 37°.

Determination of Pyridine Dinucleotides, ATP and ADP

Both NADH and NADPH were extracted from the cells using 0.5 M KOH. ATP, ADP, and NAD^+ were extracted from cells using perchloroacetic acid [18]. The pyridine dinucleotides ATP and ADP were analyzed subsequently by HPLC. Cellular extracts were separated on an RP-18, 250 × 4.5 mm column with a 0.1 M KH_2PO_4 (pH 6.5)/methanol gradient over 30 min, at a flow rate of 1 mL/min [18]. The gradient was produced by an HP 1050 series quaternary pump. An HP 1050 series diode array detector with wavelengths set at 254, 260, 280, and 340 nm was used. ATP, ADP, and NAD^+ were detected by monitoring the column effluent at wavelengths of 254, 260, and 280 nm. NADH and NADPH were detected at 340 nm. Nucleotides were identified by their retention times and quantitated by comparing the response from samples with the UV response of authentic compounds.

Measurement of Lactate and Pyruvate

Cells were washed three times with phosphate-buffered saline (pH 7.4) and centrifuged to collect the pellet. The cellular lactate and pyruvate were extracted using 8% ice-cold perchloric acid solution. Lactate released into the medium by cells was also assayed following 24 hr of (*R*)-lipoate treatment. Lactate from cells and medium was assayed as described by Tinder [19]. Pyruvate content of the cells was measured according to the procedure of Lamprecht and Heinz [20].

Determination of Cellular Glucose Uptake

Glucose uptake by cells was measured by determining the amount of glucose remaining in the culture medium following each experiment as compared with the initial glucose levels of the culture medium. Glucose was measured from the perchloric acid extracts of the medium using a Glucose Kit No. 510-DA (Sigma Chemical Co., St. Louis, MO, U.S.A.).

Protein Assay

Cells were washed three times with phosphate-buffered saline (pH 7.4) and centrifuged to collect the pellet. The cell pellet was solubilized in 0.5 N NaOH, and total protein was assayed by the method of Lowry *et al.* [21].

Statistical Analyses

Results are presented as means ± SD of three separate experiments. The differences between means of groups were compared by the Student's–Newman–Keuls test. The level of significance was set at $P < 0.05$.

RESULTS

Pyridine Dinucleotides and High-Energy Phosphates

α-Lipoate (0.5 mM) treatment of Wurzburg cells for 4 hr significantly decreased the levels of NADH compared with their controls (Fig. 1A). The tendency for NADH to decrease in α-lipoate-treated cells was apparent as early as 0.5 hr after treatment and an ~30% decrease in NADH levels was observed after 24 hr of treatment (Fig. 1A). No further decrease in NADH levels was observed in cells treated with α-lipoic acid for 48- or 72-hr periods (data not shown).

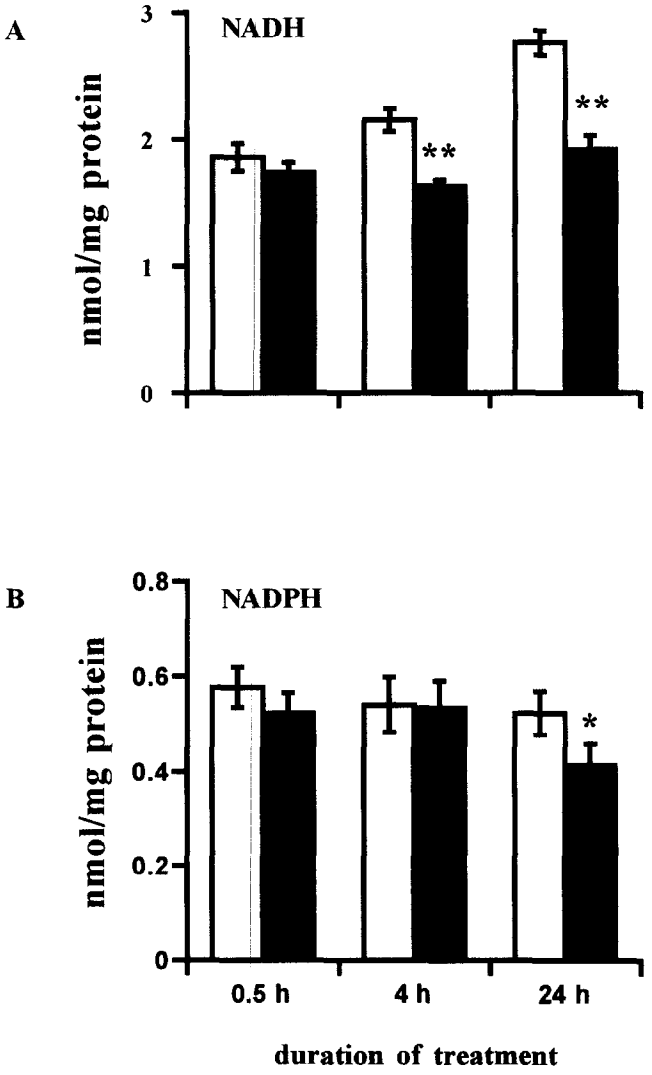

FIG. 1. Effect of 0.5 mM (*R*)-α-lipoate treatment on NADH (A) and NADPH (B) levels of Wurzburg T-cells. Open bars represent the control group and closed bars the (R)-α-lipoate acid-treated group. Data are means ± SD of three separate experiments. Key: (*) $P < 0.05$; and (**) $P < 0.001$.

NADPH levels in the cells remained unchanged up until 24 hr after α-lipoate treatment when 20% ($P < 0.05$) decreased NADPH levels were observed (Fig. 1B).

α-Lipoate treatment did not have any significant effect on NAD^+ levels in cells. ATP levels also remained unchanged following α-lipoate treatment for 24 hr. However, under these same conditions, a 25% increase in the ADP levels was observed in cells exposed to α-lipoate for 24 hr (Table 1).

Cellular Lactate and Pyruvate

The cellular content of lactic acid (Fig. 2A) and lactate release into the medium (Fig. 2B) by Wurzburg T-cells were similar in α-lipoic acid-treated and untreated groups for 24 hr, whereas during this time interval pyruvate levels in cells increased by ~25% compared with the untreated cells (Fig. 2A).

Glucose Uptake by Cells

Glucose uptake by cells treated with various concentrations (0.05 to 0.5 mM) of α-lipoate for 24 hr was determined on the basis of changes in glucose of the culture medium. The uptake of glucose by untreated cells was 0.45 mg glucose/10^6 cells per day, whereas α-lipoate-treated cells showed an increased consumption of 0.79 mg glucose/10^6 cells per day (Fig. 3). Consumption of extracellular glucose by the cells increased in a concentration-dependent manner with increasing α-lipoate concentrations (Fig. 3).

DISCUSSION

Reduction of exogenous α-lipoic acid to DHLA is known to occur in mammalian cells and tissues by mitochondrial and cytosolic enzymes that use NADH or NADPH as cofactors [13, 22]. In Wurzburg T-cells exposed to α-lipoate, NADH depletion was evident within 30 min. These results are consistent with earlier observations on the kinetics of the reduction of α-lipoic acid [13]. The *R*-enantiomer of α-lipoic acid was used in this study because (*R*)-lipoamide is the natural substrate for the dihydrolipoamide dehydrogenase enzyme and reduction of the *R*-form of α-lipoate is preferred by the enzyme as compared with the *S*-enan-

TABLE 1. α-Lipoate-dependent changes in NAD^+, ATP and ADP in human Wurzburg T-cells

Nucleotides	Control (nmol/mg protein)	R-Lipoate (nmol/mg protein)
NAD^+	6.4 ± 0.3	6.1 ± 0.2
ATP	20.4 ± 1.3	20.3 ± 1.9
ADP	6.3 ± 0.3	8.3 ± 0.7*

Cells were treated with 0.5 mM (R)-α-lipoic acid for 24 hr. Data are means ± SD of three separate experiments.

* $P < 0.05$.

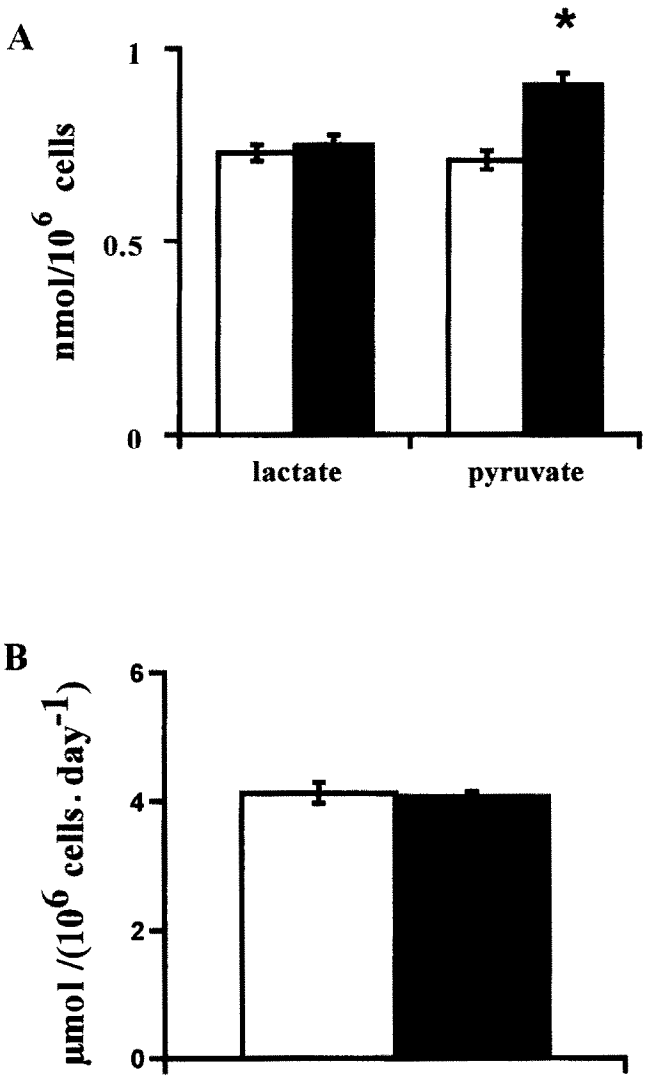

FIG. 2. Effect of 0.5 mM (*R*)-α-lipoate treatment on cellular lactate and pyruvate contents (A) and lactate released in medium. (B). Open bars represent control group and closed bars the (*R*)-α-lipoic acid-treated group. Data are means ± SD of three separate experiments. Key: (*) $P < 0.05$.

tiomer [16]. Wurzburg T-cells were selected as a model for this study because T-cell lines (Jurkat and Wurzburg) have been reported to effectively reduce lipoate to DHLA, and the enzyme systems that catalyze such reactions have been well characterized [13, 16].

Compared with that of NADH, the α-lipoate-dependent decrease in cellular NADPH was relatively less and slower. A significant lowering of cellular NADPH was observed only 24 hr after α-lipoate treatment. Two explanations may account for this observation: (i) that the major part of the reduction of α-lipoic acid to DHLA in Wurzburg cells is catalyzed by dihydrolipoamide dehydrogenase, which uses NADH as co-factor, and (ii) that NADPH also participates in the slower glutathione reductase-dependent formation of DHLA [22]. Following α-lipoic acid treatment, an early depletion of NADPH was not observed, possibly because NADH may be involved in maintaining NADPH levels

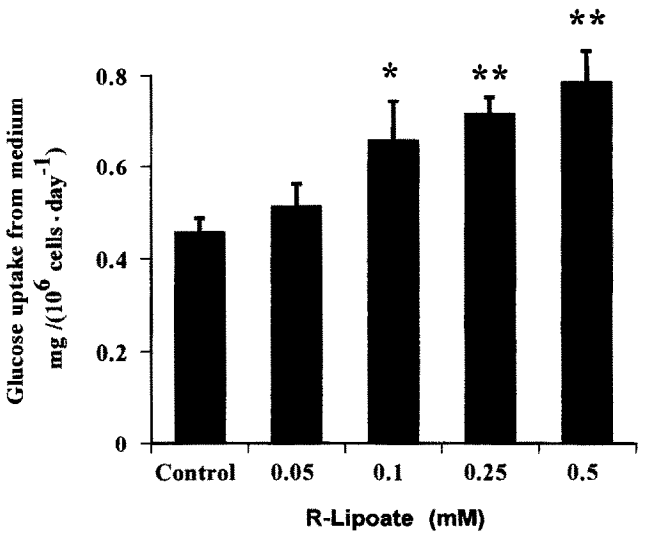

FIG. 3. Effect of different concentrations of (*R*)-α-lipoic acid treatment on extracellular glucose uptake by Wurzburg T-cells. Data are means ± SD of three separate experiments. Key: (*) $P < 0.05$; and (**) $P < 0.001$.

through transhydrogenase inter-conversion pathways. Kehrer and Lund [1] reported that in cells challenged with *tert*-butyl hydroperoxide, about half of the GSSG formed in mitochondria is reduced by NADPH regenerated from NADH through energy-dependent transhydrogenation. The mechanism and physiological significance associated with this direct cross-talk between NADH and NADPH systems are not well understood [1].

α-Lipoic acid contains a disulfide moiety, and such compounds with a disulfide moiety have been reported to stimulate NAD^+ hydrolysis [23]. Cytotoxic effects of oxidants have been proposed to be based on a severe depletion of NAD^+ followed by ATP depletion [1, 24]. DNA strand

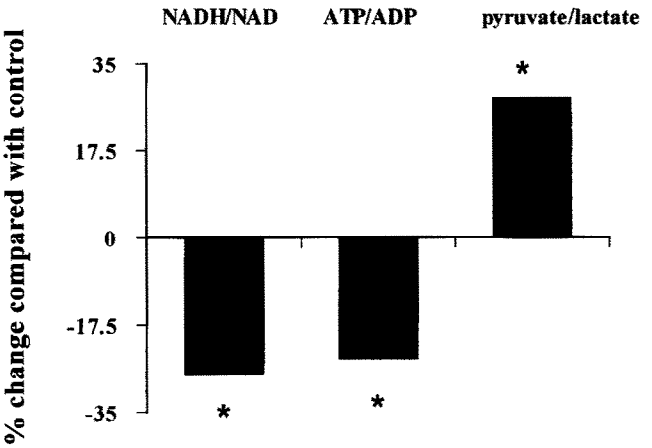

FIG. 4. Relative change in the NADH/NAD^+, pyruvate/lactate, and ATP/ADP ratios in Wurzburg T-cells treated with 0.5 mM (*R*)-α-lipoic acid compared with the untreated cells. Control values of NADH, NAD^+, pyruvate, lactate, ATP, and ADP are indicated in Table 1 and Figs. 1 and 2. Data are means of three separate experiments. Key: (*) $P < 0.05$.

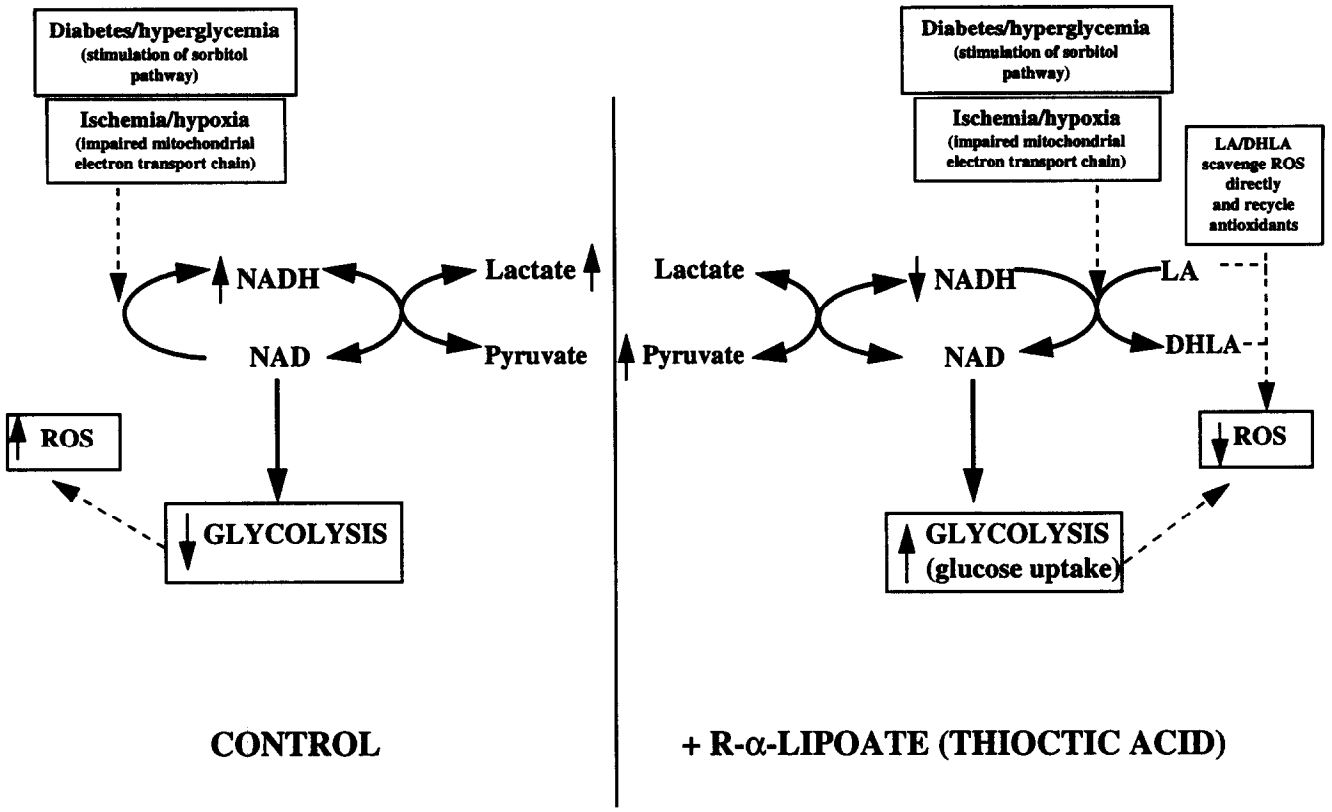

FIG. 5. Schematic representation of the influence of α-lipoic acid (LA) reduction to dihydrolipoate (DHLA) on reducing equivalent homeostasis in Wurzburg T-cells and suggested beneficial effects of such reduction in hyperglycemia and hypoxia. An increase in the NADH to NAD^+ ratio has been observed in hyperglycemia because of the increased rate of oxidation of sorbitol to fructose and in hypoxic tissue due to impaired mitochondrial electron transport. An elevated NADH/NAD^+ ratio or reductive stress in cells inhibits several major metabolic pathways, such as glycolysis and fatty acid oxidation, and promotes intracellular formation of reactive oxygen species (ROS). α-Lipoic acid decreases the NADH/NAD^+ ratio or reductive stress by rapidly consuming NADH during its reduction to DHLA. In addition, both α-lipoic acid and DHLA are potent antioxidants in that they directly scavenge ROS and recycle thioredoxin, glutathione, vitamin E, and vitamin C.

breaks following oxidant challenge activates poly(ADP-ribose) polymerase that hydrolyzes NAD^+ to provide nucleotides for DNA repair [25]. Because ATP is the immediate source of energy for most biological energy-requiring reactions, the depletion of this energy pool is a critical factor contributing to oxidant-induced cytotoxicity [24]. Our results show that 0.5 mM α-lipoate in Wurzburg cells does not deplete cellular NAD^+ or ATP significantly.

NADH is utilized primarily in energy metabolism, whereas NADPH is involved mainly in reductive biosynthesis. The ratio of the concentration of free NAD(P)$^+$ and NAD(P)H at the site of oxido-reduction is crucial because it influences the metabolic behavior of oxidized and reduced substrates, and in doing so cellular NAD(P)H/NAD(P) modulates metabolic activity [26], as summarized below in Fig. 5. α-Lipoic acid treatment decreased the ratio of free NADH to NAD^+ and increased the pyruvate to lactate ratio (Fig. 4). These findings are consistent with each other because the lactate/pyruvate ratio is known to reflect the cytosolic free NADH/NAD^+ ratio [2]. A decrease in the cellular NADH to NAD^+ ratio following treatment with α-lipoic acid may have direct implications in diabetes and other pathologies where redox imbalances, e.g. high NADH/NAD^+ ratio, are known to be one of the major causative factors [2]. Inhibition of cellular glucose uptake, glucose utilization, and glycolysis in response to a decreased supply of NAD^+ is one of the major metabolic disorders associated with diabetes and ischemia–reperfusion injury [2, 27]. The observed concentration-dependent increase in glucose uptake by Wurzburg cells treated with α-lipoate is consistent with a previous report that α-lipoate enhances glucose disposal in patients with Type II diabetes [7]. Administration of α-lipoic acid to rats has been reported to stimulate the activities of glycolytic enzyme that are inhibited by gentamicin [28].

Besides inhibiting major metabolic pathways such as glycolysis, high cellular NADH acts as a reductant and may promote production of oxygen radicals [1, 2, 4]. Formation of ROS during reductive stress caused by inhibitors of the mitochondrial respiratory chain has been reported to facilitate cell killing by attacking intracellular lipids, thiols, DNA, and other cellular components [3]. Antioxidant treatment under such conditions delays reductive stress-induced cell killing [3]. Interaction of NADH and iron in the microsomes produces oxidants capable of initiating lipid peroxidation in cells [29]. Dihydrolipoate readily scav-

enges a majority of ROS such as hypochlorous acid, and peroxyl, superoxide, hydroxyl, and nitric oxide radicals [5]. α-Lipoic acid is also reported to scavenge ROS, such as singlet oxygen, hydroxyl radical, and hypochlorous acid, directly [5]. With a redox potential of −0.32 V for the DHLA/ α-lipoic acid couple, DHLA is a strong reductant and may reduce GSSG to GSH, the redox potential of the GSH/GSSG couple being −0.24 V [30]. Treatment of human Jurkat T-cells with α-lipoic acid has been reported to increase the intracellular glutathione levels in these cells [31]. Free lipoate also promotes reduction of thioredoxin in mitochondria [32]. DHLA is known to reduce thioredoxin [33] and regenerate other major physiological antioxidants of the lipid and aqueous phase, such as vitamin E and ascorbate [21, 34].

In pathologies such as hyperglycemia, ischemia–reperfusion injury, and ethanol toxicity that have both reductive and oxidative imbalances, supplementation of α-lipoic acid may have dual beneficial effects (Fig. 5). Under the condition of reductive stress, α-lipoic acid decreases high NADH levels in the cell by utilizing it as a co-factor in the reduction process, whereas in oxidative stress situations both α-lipoic acid and its reduced form, DHLA, may protect by directly scavenging free radicals and regenerating other antioxidants from their oxidized forms. Although several beneficial antioxidant properties of α-lipoic acid have been reported previously, this work presents novel information on how lipoate treatment may alleviate reductive stress and thus be beneficial in pathologies that have redox complications.

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