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REDUCTION AND TRANSPORT OF LIPOIC ACID BY HUMAN ERYTHROCYTES

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Abstract—Reduction of exogenous lipoic acid to dihydrolipoate is known to occur in several mammalian cells and tissues. Dihydrolipoate is a potent radical scavenger, and may provide significant antioxidant protection. Because lipoic acid appears in the bloodstream after oral administration, we have examined the reduction of exogenous lipoate by human erythrocytes. Normal human erythrocytes reduced lipoate to dihydrolipoate only in the presence of glucose; deoxyglucose did not substitute for glucose, indicating that the reduction of lipoate requires glucose metabolism. Furthermore, the reduction was shown to be NADPH dependent. Erythrocytes isolated from a human subject with a genetic deficiency of glucose-6-phosphate dehydrogenase (and, therefore, deficient in the formation of NADPH) did not reduce lipoate. Dehydroepiandrosterone, a specific inhibitor of glucose-6-phosphate dehydrogenase, inhibited lipoate reduction. Our findings imply that some of the reduction of exogenous lipoic acid is catalysed by glutathione reductase, a flavoprotein dehydrogenase; mitomycin C, an inhibitor of FAD-dependent reductases, inhibited lipoate reduction by erythrocytes, and glutathione reductase purified from human erythrocytes was observed to reduce lipoic acid in a cell-free system. We further explored these findings with erythrocyte ghosts and liposomes. Our results indicate that a transport system exists for α-lipoic acid and dihydrolipoate; resealed erythrocyte ghosts, containing trapped lipoamide dehydrogenase and pyridine nucleotides, reduced externally added lipoate. By contrast, liposomes prepared with enzyme and pyridine nucleotides did not catalyze reduction of lipoate. This work indicates that uptake of exogenous lipoate and reduction to dihydrolipoate by normal human erythrocytes may contribute to oxidant protection in the human bloodstream.

Key words: erythrocytes; α-lipoic acid; dihydrolipoic acid; glutathione reductase; NADPH; glucose-6-phosphate dehydrogenase; membrane; transport

After oral administration, lipoic acid is present in the bloodstream [1]. Hence, erythrocytes, platelets, lymphocytes and other plasma constituents could be targets for its actions.

Previous studies have indicated that mammalian tissues possess metabolic pathways capable of converting lipoic acid to dihydrolipoate [2, 3]. Dihydrolipoate is an excellent reducing agent, and recent work has demonstrated the ability of exogenous dihydrolipoate to prevent oxidative damage to membranes [3] and low-density lipoproteins [4]. Dihydrolipoate shows synergy with vitamin E in protection against cardiac damage from ischemia–reperfusion [5]. Only the reduced form of lipoate was found to be effective in the protection against lipid peroxidation and vitamin E consumption [6], but both forms are effective in the protection of human erythrocytes against peroxyl radical-induced hemolysis [7, 8]. The mechanism of radical-scavenging by lipoate requires further investigation.

Until recently, the measurement of lipoic acid reduction by erythrocytes could not be accomplished. Our laboratory has developed an HPLC method [2, 9] that permits concomitant detection of minute amounts of both reduced and oxidized lipoic acid. The reduction of lipoate may account for some of the previous findings during lipoic acid incubation with erythrocytes. Furthermore, reduction of lipoic acid by erythrocytes may contribute directly to the pool of dihydrolipoate in the bloodstream. Therefore we have undertaken a detailed study of lipoate metabolism in isolated human erythrocytes. Using this newly developed HPLC method [2, 9], we have explored the transport of lipoic acid across isolated erythrocyte membranes, and enzymatic pathways for erythrocyte reduction of lipoic acid.

MATERIALS AND METHODS

Isolation of erythrocytes from whole blood

Whole blood was obtained by venipuncture from human volunteers, using heparin anticoagulant. Cells were washed three times by centrifugation for 10 min at 1075 *g* in isotonic PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4). The supernatant and buffy coat were removed carefully and discarded after each wash.

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HPLC determination of reduced and oxidized lipoate

Both lipoic acid and dihydrolipoate were measured by HPLC, with electrochemical detection, using the method of Handelman, Han and coworkers [2, 9]. Lipoic acid and dihydrolipoate were separated on a 10 cm × 0.46 cm C18 column, 3 μm particle size (Rainin Instruments, Emeryville, CA). The mobile phase was 50% H_2O (containing 2% monochloroacetic acid, pH 2.9), 30% methanol, and 20% acetonitrile. The mobile-phase flow rate was 1.0 mL/min for most analyses (although slower flow rate was sometimes used if column pressure was high). After HPLC separation, both lipoic acid and dihydrolipoate were detected with a dual Hg/Au electrode. Full details of the method are described in the article by Han *et al.* [9].

Reagents

(*RS*)-Lipoic acid and (*RS*)-dihydrolipoate were obtained from Asta Medica, Frankfurt, Germany. The enzyme inhibitors DHEA* and mitomycin C were from the Sigma Chemical Co. (St. Louis, MO).

Both lipoic acid, and dihydrolipoate, can be dissolved at a 10 mM concentration in 20 mM PBS, pH 7.4, by stirring at 25° for 1 hr. Concentrations from 0.5 to 9 mM were used for these experiments. NADPH (for experiments with glutathione reductase) and NADH (for experiments with lipoamide dehydrogenase) were obtained from Sigma. FAD was also obtained from Sigma.

Measurement of dihydrolipoate formation by erythrocytes

For direct assay of lipoic acid reduction, erythrocytes were incubated in isotonic PBS at 37° in a shaking water bath. Several reagents (lipoic acid, glucose, deoxyglucose, and inhibitors) were added to the buffer at the start of the incubation. At the end of the incubation period, the erythrocytes were pelleted in a microcentrifuge tube at 10,000 *g* for 2 min. The supernatant was then injected onto the HPLC.

Some samples could not be analyzed immediately by HPLC. Because dihydrolipoate easily reoxidizes back to lipoic acid, these samples were stabilized by mixing one part sample with one part methanol (containing 1% monochloroacetic acid), and storing the mixture at −80° until analysis. Control experiments (data not shown) indicated that dihydrolipoate and lipoate can be stored for up to 1 month under these conditions without loss.

HPLC measurement of dihydrolipoate was not possible in erythrocyte lysate, because free hemoglobin (obtained from freshly hemolyzed erythrocytes) very rapidly reoxidizes dihydrolipoate to lipoate.

Preparation of resealed ghosts

Right-side-out erythrocyte ghosts were prepared from normal human erythrocytes [10]. Washed human erythrocytes were hemolyzed in 40 vol. of 5 mM sodium phosphate, pH 8.0. The erythrocyte membranes were pelleted in the same buffer by centrifugation at 22,000 *g* for 10 min. After four washes, white ghosts were obtained.

One milliliter of concentrated ghost membranes (2 mg protein/mL) was incubated with 100 U of lipoamide dehydrogenase (porcine heart, Sigma), 2% BSA (Fraction V, defatted) and 10 mM NAD^+ or NADH for 10 min at 4°. Nine milliliters resealing medium (containing PBS, adjusted to pH 6.5, 2% BSA, 10 U/mL lipoamide dehydrogenase and 6 mM NAD^+ or NADH) was added and the membranes were incubated for 40 min at 37°. The resealed ghosts were washed three times in PBS (pH 7.6) as above and kept at 4° until used. Resealed ghosts were used within 24 hr of preparation.

Preparation of liposomes that contain enzymes and pyridine nucleotides

Trapping of lipoamide dehydrogenase and pyridine nucleotides in liposomes was achieved by the freeze/thaw method, as described previously [11]. Liposomes prepared from 14 mM dioleyl phosphatidylcholine, 7 mM cholesterol and 2 mM dicetyl phosphate were sonicated to clarity in PBS (20 min in a bath-type sonicator). The liposomes were then mixed with 2% BSA, 50 U/mL lipoamide dehydrogenase and 10 mM NAD^+ or NADH. The mixture was frozen once in liquid nitrogen, thawed, and briefly sonicated; untrapped lipoamide dehydrogenase and pyridine nucleotide were removed by centrifugation through a Sephadex G-50 column equilibrated with PBS.

Lipoamide dehydrogenase assay in erythrocyte ghosts and liposomes by NAD^+ reduction/NADH oxidation

Measurements were carried out at 37° in a Perkin–Elmer MPF-44 fluorescence spectrophotometer (Perkin–Elmer Instruments, Norwalk, CT) by following NADH fluorescence changes. In the 3-mL quartz cuvette were placed 2.5 mL of 150 mM NaCl, 10 mM sodium phosphate, pH 8, and reconstituted ghosts containing 0.5 mg erythrocyte membrane protein. Other measurements were conducted with 2 mL buffer and 0.5 mL of liposome suspension. The reaction was initiated by addition of lipoic acid or dihydrolipoic acid from 100-fold concentrated stock solutions in ethanol. Fluorescence changes were followed at excitation and emission wavelengths of 340 and 460 nm, respectively, with slits adjusted to 5 nm bandwidth. The quantity of reduced pyridine nucleotide was determined by the addition of calibrated aliquots of NADH to the same cuvette.

Lipoamide dehydrogenase assay in erythrocyte ghosts and liposomes, by HPLC measurement

The reaction was carried out in the same manner as the NAD^+/NADH interconversion described above, except that the enzymatic reaction was stopped by mixing 250 μL of sample with 250 μL of methanol containing 1% monochloroacetic acid. The samples were then stored at −80° until HPLC analysis. After thawing, the samples were centrifuged for 2 min at 12,000 *g* in a microcentrifuge, and analyzed within 5 min of thawing by HPLC, as described below. This prompt HPLC analysis

* Abbreviations: DHEA, dehydroepiandrosterone; G6P-DH, glucose-6-phosphate dehydrogenase; and GSSG, oxidized glutathione.

prevented reoxidation of dihydrolipoate in the thawed samples.

Assay of glutathione reductase activity with different substrates

GSSG substrate. Measurement with GSSG substrate was conducted by following oxidation of NADPH at 340 nm. Glutathione reductase (0.4 U/mL human erythrocyte glutathione reductase, obtained as a gift from K. Becker and R. Schirmer, Heidelberg, Germany) was preincubated with 1 mM FAD for 5 min at 37°, in 50 mM Tris–HCl, 2 mM EDTA, pH 8.0. The reaction mixture was then incubated for an additional 5 min with 3 mM GSSG. Enzymatic activity was initiated by addition of 0.1 mM NADPH. The decrease in $O.D._{340}$ was followed at 37° in a Shimadzu UV-160 spectrophotometer. Reference cuvettes contained all components except for glutathione reductase.

Lipoate substrate. Glutathione reductase was incubated with lipoic acid (3 mM) as described above, and the activity was determined from the oxidation of NADPH. Activity was also determined from the rate of formation of reduced lipoic acid (dihydrolipoate), as described above.

RESULTS

Reduction of lipoic acid by normal human erythrocytes

Human erythrocytes were incubated with lipoate (in PBS, with 5 mM glucose, pH 7.4, at 37°). A typical HPLC analysis, after incubation for 3 hr, is shown in Fig. 1 (top panel). If glucose was omitted from the incubation buffer, no dihydrolipoate was formed (Fig. 1, middle panel). If lipoic acid and glucose were incubated together without erythrocytes, there was also no reduction (Fig. 1, bottom panel).

The quantity of dihydrolipoate accumulated in the external medium increased along with the concentration of exogenous lipoate (Fig. 2, upper panel) and the cell number (Fig. 2, lower panel). The amount of dihydrolipoate reached a steady state after 2 hr, which may reflect the spontaneous reoxidation of dihydrolipoate that we have observed in previous studies [2].

Because of the very rapid oxidation of dihydrolipoate by free hemoglobin, the data reported here are restricted to measurements of dihydrolipoate released by erythrocytes into the medium.

Studies of components of NADPH-linked enzymatic pathways

When glucose (5 mM) was replaced by 2-deoxy-D-glucose (5 mM), a nonmetabolizable analog of glucose, the reaction was inhibited almost completely (Fig. 3). The erythrocyte cannot produce NADPH from 2-deoxy-D-glucose. These results suggest that reduction of lipoic acid takes place inside the cell and depends on red cell metabolism.

To further understand the reduction of lipoate by erythrocytes, we isolated erythrocytes from a subject previously diagnosed with a genetic deficiency of G6P-DH [12]. Earlier clinical studies of this subject also indicated normal levels of erythrocyte glutathione reductase. As shown in Fig. 4 (middle panel), no reduction of lipoate was observed by these G6P-DH-deficient erythrocytes.

When normal erythrocytes were treated with DHEA, a specific steroidal inhibitor of G6P-DH [13], the reduction of lipoate was inhibited by 65% (Fig. 4, lower panel). This finding provides further evidence that reduction of lipoate by human erythrocytes is dependent on production of NADPH.

HPLC retention times differ between Fig. 1 and Fig. 4, because sometimes high column pressure required the use of a lower mobile-phase flow rate.

After addition to erythrocytes of 300 μM mitomycin C, a membrane-permeable inhibitor of flavoprotein reductases [14], we observed 90% inhibition of lipoate reduction by erythrocytes (Fig. 5). Since glutathione reductase is a flavoprotein, the effects of mitomycin C support a role for glutathione reductase in the reduction of lipoate.

To test whether lipoate reduction in erythrocytes may indeed be catalyzed by glutathione reductase, the activity of purified human erythrocyte glutathione reductase was measured with lipoic acid substrate. The data in Table 1 indicate that purified erythrocyte glutathione reductase catalyzed lipoic acid-dependent NADPH oxidation at 1% of the rate measured with oxidized glutathione substrate, in approximate agreement with Icén [15]. Glutathione reductase also reduced lipoic acid to dihydrolipoate, as determined by HPLC detection of dihydrolipoate (Table 1).

The rate of reduction estimated by HPLC measurement of dihydrolipoate was 25% less than the rate estimated from NADPH oxidation. This difference may be due to the instability of dihydrolipoate, which partially re-oxidized back to lipoic acid before the HPLC measurement of dihydrolipoate was accomplished.

Transport of lipoic acid in erythrocyte ghosts and liposomes

The experiments described above indicated that intact, normal human erythrocytes will convert exogenous lipoic acid to dihydrolipoate, if incubated at 37° with physiological glucose levels (5 mM). Since isolated human erythrocyte glutathione reductase was also capable of reducing lipoic acid, we sought to determine if lipoic acid and dihydrolipoate could pass through erythrocyte membranes, and liposomal membranes. We therefore examined transport with ghost membranes prepared from human erythrocytes, and with liposomal membranes prepared from phospholipids. We hypothesized that liposomal membranes (which are prepared without membrane proteins) would lack a membrane component necessary for lipoic acid transport.

These studies of membrane uptake were conducted with the mitochondrial enzyme lipoamide dehydrogenase, and either NADH or NAD^+ cofactor. Lipoamide dehydrogenase is completely absent, or present at low levels, from cells that lack mitochondria (such as erythrocytes). However, lipoamide dehydrogenase has much higher activity with lipoate and dihydrolipoate substrate than does glutathione reductase [15], and therefore provides a convenient experimental tool for studies of membrane transport of these compounds.

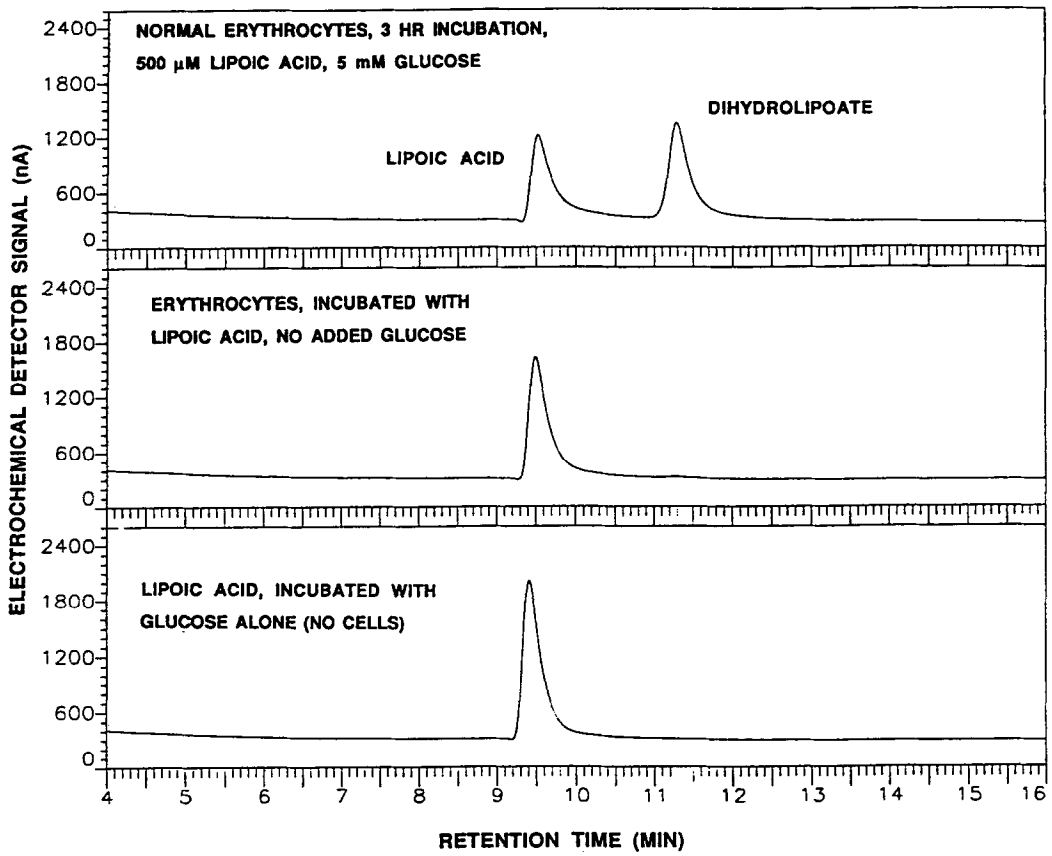

Fig. 1. HPLC analysis of dihydrolipoate released by erythrocytes during incubation with lipoic acid; in normal human erythrocytes incubated with glucose (upper trace), control erythrocytes without glucose (middle trace), control sample with glucose but without cells (lower trace). Hematocrit = 10%. T = 37°. HPLC was done on a C18-3 μm column (Rainin Instruments), 10 cm × 0.46 cm. Mobile phase: 50% H_2O/30% methanol/20% acetonitrile, 1% monochloroacetic acid, flow rate = 0.8 mL/min. Electrochemical detection was accomplished with a dual Hg/Au electrode.

To evaluate the possibility that reduction of lipoic acid was carried out at the membrane surface, we incubated washed erythrocyte ghosts with lipoic acid, and with either NADPH or NADH, and no reduction of lipoic acid was observed.

Because these preparations of resealed ghosts and liposomes contained varying amounts of lipoamide dehydrogenase, there was substantial variation in the absolute enzymatic rates between preparations. However, the trends shown here (erythrocyte membranes can transport lipoate and dihydrolipoate, but artificial liposomes cannot accomplish this transport) were seen consistently in different preparations. Each experiment was conducted several times, and results of individual representative experiments are shown.

Human erythrocyte ghosts. Transport of lipoic acid and dihydrolipoate across the erythrocyte membrane was monitored with resealed ghosts. The resealed ghosts (with trapped enzyme and substrate) were examined by HPLC measurement for reduction or oxidation of externally added lipoate or dihydrolipoate. Rates of reaction of encapsulated enzyme and pyridine nucleotide with externally added substrate are shown in Table 2. For the experiment shown here, the oxidation of dihydrolipoate (with accompanying formation of NADH) occurred 5-fold more rapidly than the reverse reaction. Although the amount of enzyme encapsulated/quantity of erythrocyte ghosts varied somewhat between preparations, we consistently observed that purified lipoamide dehydrogenase was more active at the oxidation of dihydrolipoate than the reduction of lipoate.

The rates of reaction with externally added lipoate and dihydrolipoate were not affected by addition of Trion X-100 to the erythrocyte ghost preparations (Table 2), indicating that the membrane transport system is not rate-limiting for these reactions inside the resealed ghosts.

Demonstration that erythrocyte ghost membranes are tightly sealed. In the previous experiment, lipoic acid added to the erythrocyte ghost suspension would be readily reduced if lipoamide dehydrogenase and NADH were to leak out of the ghosts, with concomitant oxidation of NADH. As a control, we added lactic dehydrogenase and pyruvate to the solution in which these ghosts were suspended, and

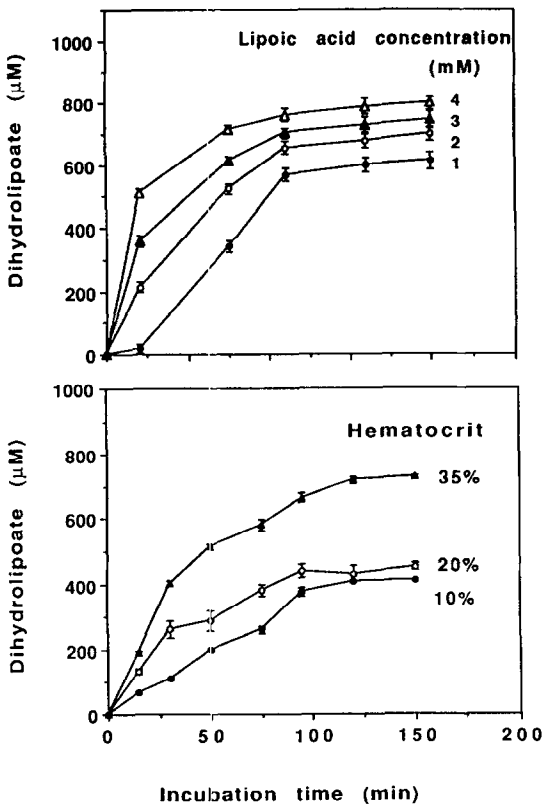

Fig. 2. Effects of lipoic acid concentration (upper panel) and packed cell volume (lower panel) on lipoic acid reduction by erythrocytes. Dihydrolipoate released from the erythrocytes, was measured in the incubation buffer. Lipoic concentration, for lower panel, was 500 μM. Values (means ± SEM) are calculated from five experiments.

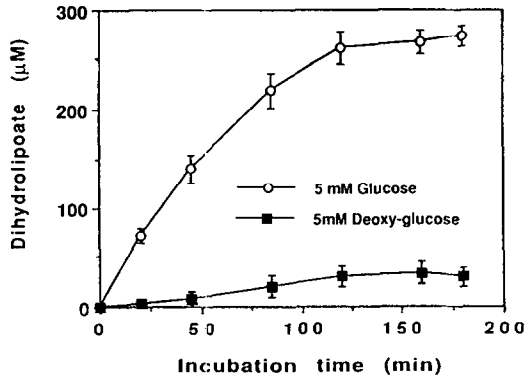

Fig. 3. Effects of glucose and deoxyglucose on lipoic acid reduction by normal erythrocytes. Hematocrit = 20%. T = 37°. Lipoic acid = 500 μM. Values (means ± SEM) are calculated from five experiments.

examined for loss of the fluorescence of the encapsulated NADH. The NADH fluorescence signal remained stable, and then dropped rapidly after the ghosts were disrupted with 1% Triton X-100 (data not shown). This finding indicates that the erythrocyte ghosts are well-sealed.

Transport of lipoic acid and dihydrolipoate by artificial liposomes. When liposomes (prepared only from phosphatidylcholine and other lipids, without membrane proteins) were examined, the results were markedly different from those obtained with resealed erythrocyte ghosts. Artificial liposomes were prepared with encapsulated lipoamide dehydrogenase and NADH. Upon addition of lipoic acid (1 mM), there was no decrease of NADH fluorescence. Even higher levels of lipoic acid had little effect on loss of NADH, but addition of 1% Triton X-100 led to a rapid degradation of the NADH signal, expressed as nanomoles NADH oxidized/per minute (Fig. 6, upper panel).

In the converse experiment, addition of dihydrolipoate to liposomes containing lipoamide dehydrogenase and NAD^+ did not lead to any increase of fluorescence until Triton X-100 was added (Fig. 6, lower panel). These findings are in marked contrast to the observations with resealed erythrocytes, where enzymatic action on externally added lipoate and dihydrolipoate occurred without addition of Triton X-100 (Table 2).

At 6 mM lipoate, and 9 mM dihydrolipoate, there was a small increase in activity of the enzyme system, which may be due to mild detergent effects at these high concentrations of lipoate and dihydrolipoate. However, the liposomal membrane remained essentially intact, as seen with the great increase in enzymatic rates after addition of 1% Triton X-100 to the liposomes.

DISCUSSION

Limited knowledge is available on the uptake and metabolism of lipoate in cells and tissues. Erythrocytes possess carriers for membrane-transport of fatty acids; Bojesen and Bojesen [16, 17] have described carrier-mediated transport of oleic acid across erythrocyte membranes, which might use the same transport process as that described here for lipoic acid. In rat hepatocytes, lipoate transport may employ the same carrier as used by medium-chain fatty acids [18]. Further studies of different fatty acid carriers are needed in erythrocytes, in relation to lipoate transport.

These findings indicate that the erythrocyte membranes have transport systems for both lipoic acid and dihydrolipoate. After addition of either lipoic acid or dihydrolipoate to resealed ghosts, both are metabolized by trapped lipoamide dehydrogenase (Table 2). When intact erythrocytes are incubated in medium containing lipoic acid, there is glucose-dependent conversion to dihydrolipoate, suggesting transfer of lipoate across the membrane to allow reduction by NADPH-dependent enzymes. These results imply that lipoate is taken up into the cells and reduced to dihydrolipoate which is, in turn, transported out into the medium. Liposomes prepared only from purified lipids did not transport

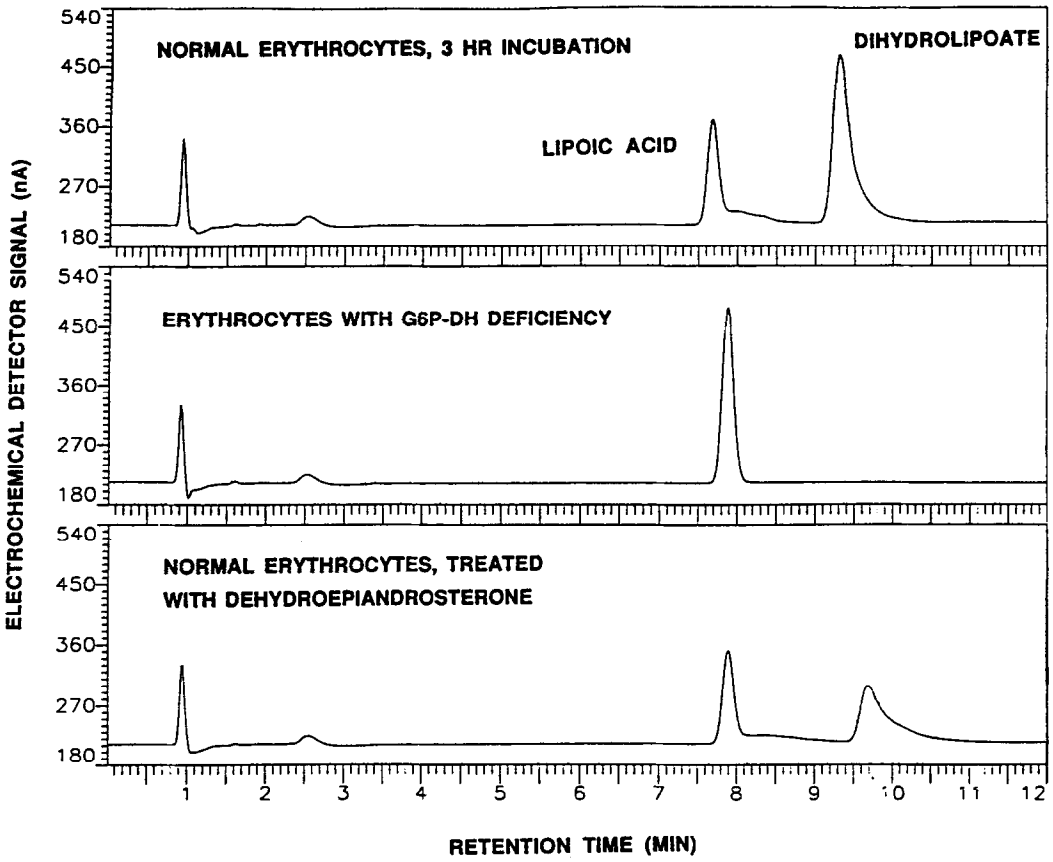

Fig. 4. Lipoic acid reduction by normal erythrocytes (upper trace), cells from a subject deficient in erythrocyte glucose-6-phosphate dehydrogenase (middle trace), and normal erythrocytes treated with dehydroepiandrosterone (lower trace). For HPLC conditions, see Fig. 1, except that flow rate = 1.0 mL/min. Hematocrit = 20%. T = 37°. Lipoic acid = 500 μM.

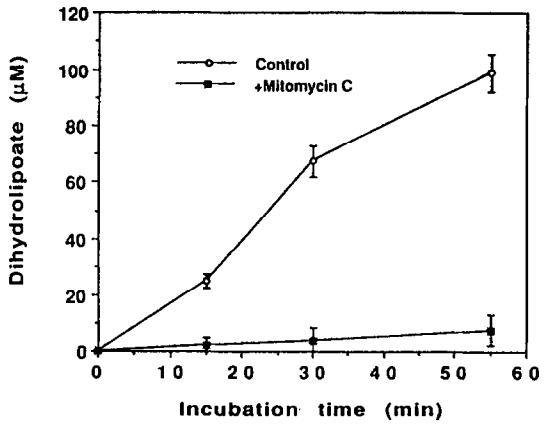

Fig. 5. Effect of mitomycin C (300 μM) on lipoate reduction by normal erythrocytes. Values (means ± SEM) are calculated from 3 experiments. Hematocrit = 20%. T = 37°. Lipoic acid = 500 μM.

lipoate (Fig. 6), indicating that the phospholipid bilayer is an effective barrier to the passage of lipoate, and that a carrier of some kind is required for lipoate transport.

Although these results were obtained with 500 μM lipoate, we observed recently (Han and Packer, manuscript in preparation) that 100 μM lipoate was reduced to dihydrolipoate and excreted by cultured human lymphocytes, suggesting that these processes can occur at lower levels than the results reported here.

Normal human erythrocytes, incubated with glucose and lipoic acid, consistently generated dihydrolipoate, which accumulated in the extracellular medium. This investigation highlights the role of glutathione reductase as a major component of lipoate reduction by normal erythrocytes. Carlberg and Mannervik [19] have calculated that NADH cofactor, at physiological pH, can only support glutathione reductase activity at 1% of the rate found with NADPH cofactor. Lack of cellular NADPH would lead to depression in all activities catalyzed by glutathione reductase. This is consistent with our observation that human erythrocytes deficient in G6P-DH, but with normal levels of

Table 1. Activity of human erythrocyte glutathione reductase with lipoic acid or oxidized glutathione as substrate

Substrate	Assay method	Reaction rate [nmol·min^{-1}·(μL enzyme)$^{-1}$]
Lipoic acid	NADPH oxidation	5.4 ± 0.4*
Lipoic acid	HPLC analysis of dihydrolipoate	3.8 ± 0.6
GSSG	NADPH oxidation	450 ± 40

* Mean ± SD, N = 4.

Table 2. NADH oxidation and NAD^+ reduction by lipoamide dehydrogenase encapsulated in human erythrocyte ghosts

Encapsulated in ghosts	Added to medium	NADH oxidation [nmol·(mg protein)$^{-1}$·min^{-1}]
(A) Lipoate-dependent NADH oxidation		
LipDH + NADH	Lipoate (2 mM)	3.1
LipDH + NADH	No additions	0.5
NADH	Lipoate (2 mM)	0.25
LipDH + NADH	DHLA (2 mM)	0.65
LipDH + NADH	Lipoate + TX-100	4.3
(B) DHLA-dependent NAD^+ reduction		
Encapsulated in ghosts	Added to medium	NAD^+ reduction [nmol·(mg protein)$^{-1}$·min^{-1}]
LipDH + NAD^+	DHLA (2 mM)	14.4
LipDH + NAD^+	No additions	0.8
NAD^+	DHLA (2 mM)	0.6
LipDH + NAD^+	Lipoate (2 mM)	1.2
LipDH + NAD^+	DHLA + TX-100	14.8

Abbreviations: LipDH, lipoamide dehydrogenase; DHLA, dihydrolipoate; and TX-100, Triton X-100, 1.0%.

glutathione reductase, lacked the ability to generate dihydrolipoate from lipoate (Fig. 4, middle panel).

Reduction of lipoic acid, and its release to the medium, was observed previously with cultured human lymphocytes and primary fibroblasts [2], but the rate of reduction was 10-fold higher in these cells than in erythrocytes. Lipoamide dehydrogenase is part of mitochondrial α-ketoacid-dehydrogenase complexes [1, 20], and reduction of exogenous lipoate by isolated mitochondria has been observed (unpublished results). In lymphocytes and fibroblasts, mitochondrial enzymes may contribute significantly to reduction of lipoate.

Erythrocytes do not possess mitochondria, but residual lipoamide dehydrogenase may be present at very low levels. However, lipoamide dehydrogenase has very high specificity for (*R*)-lipoic acid; in other experiments (data not shown), human erythrocytes reduced both (*R*)-lipoic acid and (*S*)-lipoic acid at similar rates, suggesting that much of the enzymatic activity in erythrocytes stems from an enzyme other than lipoamide dehydrogenase.

Enzymes such as glutathione reductase, thioltransferase and G6P-DH were shown previously to have a role in erythrocyte sulfhydryl homeostasis [21]. Glutathione reductase is a candidate for lipoate reduction in erythrocytes for the following reasons:

1. Glutathione reductase consumes a portion of NADPH in erythrocytes and therefore should be largely dependent on the activity of G6P-DH. This is consistent with the present results, in which low erythrocyte levels of G6P-DH (either intrinsic or produced by an inhibitor) were associated with depressed erythrocyte reduction of lipoic acid.
2. Mitomycin C, an inhibitor of GSSG reductase [14], inhibited lipoate reduction by erythrocytes (Fig. 5).
3. Isolated human erythrocyte glutathione reductase reduces lipoate to dihydrolipoate, as demonstrated here and in an earlier report [15]. The expected rate of lipoate reduction by erythrocyte glutathione reductase was calculated by assuming an erythrocyte glutathione reductase activity of 6 IU of GSSG reductase/g Hb [22] and 1% of this activity with lipoate substrate (Table 1). This leads to an estimated erythrocyte activity with lipoate of 60 nmol·(g Hb)$^{-1}$·min^{-1}, which is a significant component of the observed rate of dihydrolipoate production in intact erythrocytes, 180 nmol·(g Hb)$^{-1}$·min^{-1} (Fig. 2). However, other NADPH-dependent flavoprotein reductases may also contribute to lipoate reduction by erythrocytes.

Since both NADPH generation and glutathione

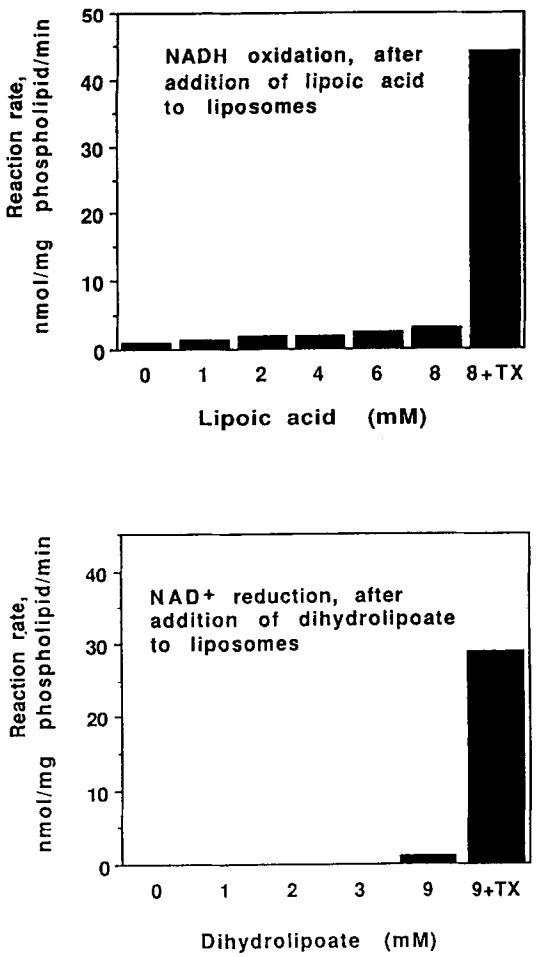

Fig. 6. Rates of pyridine nucleotide interconversion by liposomes, after addition of different concentrations of lipoate (upper panel) and dihydrolipoate (lower panel) to the external medium, and then addition of Triton X-100 to permeabilize the liposomes.

reductase activity are ubiquitous in mammalian tissues, these findings indicate that all tissues should demonstrate some reduction of lipoic acid. However, the modest amount of lipoate released by red cells (compared with several other types of cells) indicates the possibility that other cells have additional mechanisms for reduction of lipoate, and we are exploring this question. The differences in transport and reduction of (*R*)-lipoic acid and (*S*)-lipoic acid also require further investigation.

The release of dihydrolipoic acid by normal erythrocytes highlights the potential significance of dihydrolipoate in the circulation of animals given dietary lipoate. Dihydrolipoate might, for example, confer significant antioxidant protection in the bloodstream of such animals.

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