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R-Lipoic Acid Inhibits Mammalian Pyruvate Dehydrogenase Kinase

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The four pyruvate dehydrogenase kinase (PDK) and two pyruvate dehydrogenase phosphatase (PDP) isoenzymes that are present in mammalian tissues regulate activity of the pyruvate dehydrogenase complex (PDC) by phosphorylation/dephosphorylation of its pyruvate dehydrogenase (E1) component. The effect of lipoic acids on the activity of PDKs and PDPs was investigated in purified proteins system. R-lipoic acid, S-lipoic acid and R-dihydrolipoic acid did not significantly affect activities of PDPs and at the same time inhibited PDKs to different extents (PDK1 > PDK4 ~ PDK2 > PDK3 for R-LA). Since lipoic acids inhibited PDKs activity both when reconstituted in PDC and in the presence of E1 alone, dissociation of PDK from the lipoyl domains of dihydrolipoamide acetyltransferase in the presence of lipoic acids is not a likely explanation for inhibition. The activity of PDK1 towards phosphorylation sites 1, 2 and 3 of E1 was decreased to the same extent in the presence of R-lipoic acid, thus excluding protection of the E1 active site by lipoic acid from phosphorylation. R-lipoic acid inhibited autophosphorylation of PDK2 indicating that it exerted its effect on PDKs directly. Inhibition of PDK1 by R-lipoic acid was not altered by ADP but was decreased in the presence of pyruvate which itself inhibits PDKs. An inhibitory effect of lipoic acid on PDKs would result in less phosphorylation of E1 and hence increased PDC activity. This finding provides a possible mechanism for a glucose (and lactate) lowering effect of R-lipoic acid in diabetic subjects.

Keywords: Pyruvate dehydrogenase complex; Pyruvate dehydrogenase kinase isoenzymes; Pyruvate dehydrogenase phosphatase; Lipoic acid; Phosphorylation; Diabetes

INTRODUCTION

R(+)-Lipoic acid (R-LA), and not its stereoisomer S(−)-lipoic acid (S-LA), serves as a prosthetic group of several multienzyme complexes of oxidative metabolism, namely pyruvate dehydrogenase complex (PDC), branched-chain α-keto acid dehydrogenase complex, α-ketoglutarate dehydrogenase complex, and the glycine cleavage system.[1,2] R-LA is covalently attached to these enzymes through a specific lysine residue. During catalysis the lipoyl moiety transfers acyl groups and reducing equivalents between the active sites of the component enzymes within the multienzyme complexes. R-LA is synthesized in mammalian cells and is also derived from foods.[3–5] Free R-LA and its reduced form dihydrolipoic acid (R-DLA) function as antioxidants that scavenge reactive oxygen species and recycle other antioxidants such as vitamins E and C, ubiquinol and glutathione.[1,6–8] Being a coenzyme and antioxidant makes R-LA an attractive agent for therapeutic purposes.[9] R-LA was shown to increase glucose uptake by activating the insulin-signaling pathway[10–12] and has been recently used for the treatment of diabetic patients.[9,13,14]

PDC is critical for glucose oxidation, linking glycolysis with the tricarboxylic acid cycle. PDC catalyzes oxidative decarboxylation of pyruvic acid with formation of carbon dioxide, acetyl-CoA and NADH. PDC is composed of three catalytic components: pyruvate dehydrogenase (E1) catalyzing decarboxylation of pyruvic acid and reductive acetylation of lipoyl moieties of dihydrolipoamide acetyltransferase (E2); E2 catalyzing transacetylation reaction with formation

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of acetyl-CoA; and dihydrolipoamide dehydrogenase (E3) reoxidizing the lipoyl moieties of E2 with production of NADH.[15] Mammalian PDC is a highly organized multienzyme complex, in which E2 and dihydrolipoamide dehydrogenase-binding protein (BP) form the central core of the complex to which all other components are non-covalently bound.[16] E1 is bound through the E1-binding domain of E2, and E3 through the subunit-binding domain of BP. Activity of mammalian PDC is regulated by reversible phosphorylation-dephosphorylation of three specific serine residues of E1, catalyzed by a family of specific pyruvate dehydrogenase kinases (PDKs) and phospho-pyruvate dehydrogenase phosphatases (PDPs).[17–20] Four isoenzymes of PDK and two isoenzymes of PDP were identified in mammalian tissues with different catalytic and regulatory properties as well as specific tissue distributions.[20,21] The critical role played by PDC in glucose homeostasis is reflected in the complexity of its regulation.[22,23]

Insulin-mediated signaling pathways increase both the uptake of glucose by enhancing translocation of glucose transporter 4 onto the plasma membrane[11,12,24] and the level of the dephosphorylated-active form of PDC by stimulating the PDP activity in the mitochondria.[25,26] Since it has been shown that R-LA increased glucose uptake in L6 muscle cells and 3T3-L1 adipocytes,[11,12,24] LA elevated the PDC activity in liver mitochondria from diabetic rats,[25] and R-LA increased the activation state of PDC in cultured rat hepatocytes,[26] we wondered whether the effect of LA on PDC was also through the activation of PDP or via another mechanism. Our results show that lipoic acids do not activate PDPs, instead inhibit PDKs and hence reduce the phosphorylation state of PDC (increasing its activity).

MATERIALS AND METHODS

Materials

Ni-nitrilotriacetate (Ni-NTA)-agarose was from Qiagen. The protein assay reagent was obtained from BioRad. [γ-^{32}P]ATP was from ICN. Trometh-amine salt of R-LA ($C_{12}H_{25}NO_5S_2$), S-LA ($C_8H_{14}O_2S_2$) and R-DLA ($C_8H_{16}O_2S_2$) were gifts from Dr Mathias Locher and Dr Claudia Wicke of Asta Medica, Germany. Pyruvamide was kindly provided by Dr Frank Jordan of Rutgers State University. pPDK1, pPDK2, pPDK3, pPDK4, pET-28a-PDP1, and pET-28a-PDP2 expression vectors and *Escherichia coli* strain BL21 with pPDHE2/E3BP were a generous gift from Dr Robert A. Harris of Indiana University School of Medicine.

Overexpression and Purification of PDC Components

Recombinant human wild-type E1 was overexpressed in M15 *E. coli* cells harboring pQE-9-6HE1α/E1β, coexpression vector carrying coding sequences of both human E1α and E1β cDNAs and purified using Ni-NTA-agarose chromatography as previously described.[27] Several mutant human E1s having only one site for phosphorylation [E1–S2A/S3A with serines at site 2 (Ser271) and site 3 (Ser203) replaced with alanine; E1–S1A/S3A with serines at sites 1 (Ser264) and 3 replaced with alanine; E1–S1A/S2A with serines at sites 1 and 2 replaced with alanine] were constructed previously.[28] Recombinant human E2-BP was overexpressed and purified from BL21 cells harboring pPDHE2/E3BP as described previously.[29] Recombinant human E3 was overexpressed and purified to about 96% purity as described by Liu *et al.*[30] Recombinant rat PDK1, rat PDK2, human PDK3 and rat PDK4 were overexpressed and purified individually from BL21 cells with specific pPDK expression vectors to 90–95% as described.[21,29] Recombinant rat PDP1 and PDP2 were purified in the same way as PDKs. Protein was measured by the Bradford method using bovine serum albumin as the standard.[31]

Determination of PDK Activity

Activity of PDK was determined with reconstituted PDC or with E1 alone by measuring the incorporation of ^{32}P from [γ-^{32}P]ATP into E1.[32,33] To determine PDK activity in PDC, wild-type or mutant E1s (5 μg), E2–BP (5.8 μg) and E3 (2.5 μg) were incubated with specific PDK isoenzyme (0.1 μg) at 30°C for 5 min before the reaction was started with 100–500 μM [γ-^{32}P]ATP (200–1000 cpm pmole^{-1}). PDC was reconstituted from its catalytic components E1/E2–BP/E3 in the following ratio: 30 E1 tetramers/1 E2 60-mer with at least 12 BP/24 E3 dimers (amount of BP varied in the preparations of E2–BP from 12 to 24 monomers of BP per 60-mer of E2). PDK activity with E1 alone was measured using 10 μg E1. Concentrations of ATP used were [based on K_m values for ATP[33]]: 300 μM for PDK1 and PDK2, 100 μM for PDK3, and 500 μM for PDK4. Reactions were performed in a phosphate buffer system: 20 mM potassium phosphate buffer, pH 7.0, 0.1 mM EDTA, 1 mM $MgCl_2$, 2 mM dithiothreitol. Reactions were stopped at different time intervals by applying aliquots (20 μl) on paper discs presoaked in 10% trichloroacetic acid and 10 mM pyrophosphate, and the radioactivity incorporated in the protein was measured.[33]

To measure inhibitory effects of lipoic compounds on activity of PDK stimulated by reduction or

reduction/acetylation of the lipoyl moieties of E2, reconstituted PDC was incubated for 5 min with: (i) $NAD^+/NADH = 200/1$ (796 μM/4 μM) with 50 μM CoA (to generate oxidized lipoyl moieties); (ii) $NADH/NAD^+ = 3/1$ (600 μM/200 μM) with 50 μM CoA (to generate reduced lipoyl moieties); and (iii) $NADH/NAD^+ = 3/1$ (600 μM/200 μM) with 50 μM acetyl-CoA (to generate reduced and acetylated lipoyl moieties) prior to starting PDK reaction by the addition of [γ-^{32}P]ATP.[21,34]

Kinetic parameters of PDKs were determined in reactions with PDC by varying the concentrations of E1 from 0.02 to 3.89 μM. Apparent $S_{0.5}$ values were determined (and not K_m values because of cooperative interaction of E1 with PDKs) using the Hill equation: $\{v_0/E_0 = (k_{cat} \cdot S^n)/[(S_{0.5})^n + S^n]\}$. Aliquots were withdrawn from the reactions in triplicate–quadruplicate every 30 s or 1 min. The velocity of the reaction was linear with time (at least 5–10 min). Negative controls (without PDK) were subtracted from all experimental data. One unit of PDK activity is defined as 1 μmole of ^{32}P incorporated in E1 per min at 30°C. Specific activity of PDK is expressed as milliunits per mg of PDK. Correlation coefficients in all kinetic experiments were at least 95%.

Inhibition of PDK

To study the effect of lipoic compounds on PDK activity, 0–5 mM lipoic acids were added before the reactions were started by the addition of ATP. R-LA (tromethamine salt) was dissolved in water to 50 mM. S-LA and R-DLA were dissolved in 100 mM *Tris*-HCl, pH 7.0. Different amounts of 100 mM *Tris*-HCl, pH 7.0 were added to the reactions to reach the same concentration of Tris after different additions of the S-LA and R-DLA because activities of PDKs are affected by Tris concentration. The concentration of R-DLA was determined by reduction of 5,5′-dithiobis(2-nitrobenzoic acid) ($\varepsilon_{412} = 27,200\,M^{-1}\,cm^{-1}$). To investigate the effect of pyruvate or ADP on inhibition of PDK with R-LA, inhibitors were added simultaneously before addition of ATP. In these experiments reactions were performed with the oxidized lipoyl moieties of E2 only.

Autophosphorylation of PDK2

To study the effect of R-LA on autophosphorylation of PDK2, 0–5 mM R-LA was added to 7 μg of PDK2 in phosphate buffer (as described above) and reactions were started with [γ-^{32}P]ATP. Aliquots were withdrawn at different times (from 0 to 120 min) to determine radioactivity incorporated in PDK2.

Determination of PDP Activity

Activity of PDP1 and PDP2 was determined in the absence or presence of R-LA, S-LA and R-DLA by dephosphorylation of E1 phosphorylated at site 1, site 2 or site 3 by PDK2 (sites 1 and 2) and PDK1 (site 3).[28,35] E1 mutants (15 μg or 50 μg) were phosphorylated by PDK1 or PDK2 (0.33 μg) in the presence of E2–BP and 2 mM [γ-^{32}P]ATP in 150 μl reaction for 100 min as described above. ATP was depleted by incubation with 10 mM glucose and 15 μg of yeast hexokinase for 30 min. Dephosphorylation reactions were performed in the presence of 10 mM $MgCl_2$, 1 mM $CaCl_2$ and 4.4 mM dichloroacetate for PDP1 and 20 mM $MgCl_2$, 1.2 mM $CaCl_2$ and 4.4 mM dichloroacetate for PDP2. Lipoic acids were added to dephosphorylation reaction at 0–5 mM. Reactions were started by PDP1 (5 ng μl^{-1} for site 1 and 1 ng μl^{-1} for sites 2 and 3) or PDP2 (4 ng μl^{-1} for sites 1 and 3 ng μl^{-1} for sites 2 and 3). Aliquots were removed every minute untill 10 min, spotted on a paper disc and incorporated radioactivity was measured as described above for determination of PDK activity. PDP activity was calculated from the linear part of the progress curves. Negative controls (without PDK) were subtracted from all experimental data. One unit of PDP activity is defined as 1 μmole of ^{32}P released from E1 per min at 30°C. Specific activity of PDP is expressed as milliunits per mg of PDP. Correlation coefficients in all kinetic experiments were at least 95%.

RESULTS

Effect of Lipoic Acids on PDP1 and PDP2 Activity

Activities of PDP1 and PDP2 were determined by the dephosphorylation of E1 mutants phosphorylated at sites 1, 2, or 3 individually. Figure 1 shows the effect of R-LA, S-LA and R-DLA (taken at 0, 1, 3 and 5 mM) on the activity of PDP1 towards phosphorylation site 1, site 2 and site 3 of E1. The results indicate that the activity of PDP1 towards the three phosphorylation sites did not change significantly in the presence of different concentrations of R-LA, S-LA and R-DLA. The data presented in Fig. 1 were obtained at lower than saturating concentration of E1 (0.1 mg ml^{-1}). Experiments performed at higher (saturating) concentrations of E1 mutants (0.33 mg ml^{-1}) did not demonstrate any effect of R-LA on the activity of PDP1 (Table I).

Unlike PDP1, PDP2 was inhibited by R-LA, S-LA and R-DLA to similar extents as can be seen in Fig. 1 (for 0.1 mg E1/ml) and in Table I (for 0.33 mg E1/ml). Lipoic acids did not cause the activation of the PDP isoenzymes activities, but rather were inhibitory for PDP2 with low efficiency.

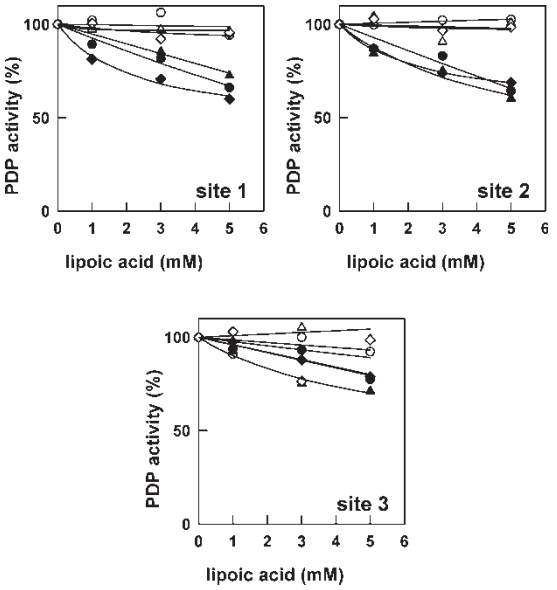

FIGURE 1 Effect of lipoic acids on activities of PDP1 and PDP2. Activities of PDPs were determined by dephosphorylation of E1 (0.1 mg ml^{-1}) reconstituted in PDC and phosphorylated at site 1, site 2 or site 3 as indicated. Lipoic acids (0–5 mM) were added during phospho-E1 dephosphorylation by PDP1 (●, R-LA; ▲, S-LA and ◆, R-DLA) and PDP2 (○, R-LA, Δ, S-LA and ◇, R-DLA). Hundred percent of PDP1 activity corresponded to: 9.4 mU mg^{-1} for site 1, 18.0 mU mg^{-1} for site 2 and 16.7 mU mg^{-1} for site 3. Hundred percent of PDP2 activity corresponded to: 5.5 mU mg^{-1} for site 1, 22.7 mU mg^{-1} for site 2 and 12.6 mU mg^{-1} for site 3. The correlation coefficients were at least 95%. Similar results were obtained for 2–3 independent experiments.

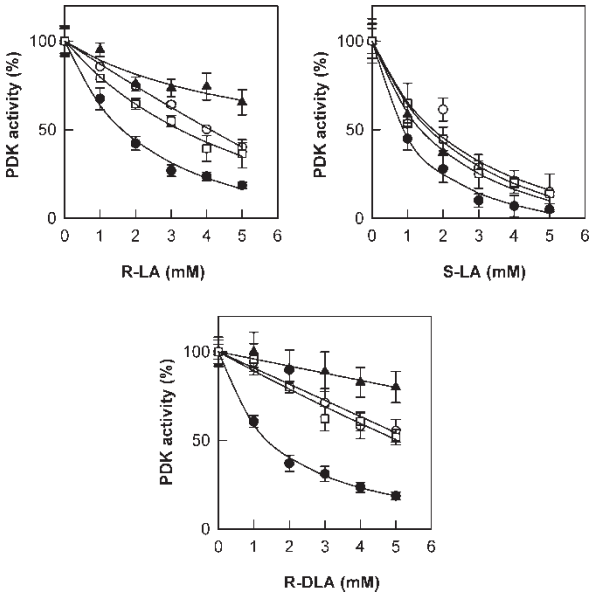

FIGURE 2 Effect of lipoic acids on activities of PDK isoenzymes. Activities of PDKs were determined in the presence of 0 to 5 mM lipoic acids as indicated with reconstituted PDC by ^{32}P incorporation into protein. Lipoyl moieties of E2 were oxidized by preincubation of reconstituted PDC with NAD$^+$/NADH = 796 μM/4 μM plus 50 μM CoA. Hundred percent of activity corresponded to: 56 mU mg^{-1}, PDK1 (●); 87 mU mg^{-1}, PDK2 (○); 64 mU mg^{-1}, PDK3 (▲); and 38 mU mg^{-1}, PDK4 (□). Results are means ± S.D. of 3–4 determinations.

Effect of Lipoic Compounds on Phosphorylation of PDC

Four isoenzymes of PDK were tested as potential targets for lipoic acids. Activities of PDKs were determined by phosphorylation of E1 reconstituted in PDC or in a free form. PDKs are bound in PDC to the lipoyl domains of E2 and BP.[22] PDKs also catalyze phosphorylation of free E1, however, binding to E2 and BP increases activities of PDKs in an isoenzymes-specific and phosphorylation site-specific manner.[32,33] Figure 2 shows the effects of R-LA, S-LA and R-DLA on the activities of PDK1, PDK2, PDK3 and PDK4 present in PDC with lipoyl moieties of E2 oxidized in the presence of NAD$^+$/NADH = 200/1. The maximum inhibition was seen for S-LA for the four isoenzymes of PDK (50% inhibition reached at 0.85 mM for PDK1, 1.73 mM for PDK2, 1.38 mM for PDK3 and 1.62 mM for PDK4). The oxidized and reduced forms of R-LA (R-LA and R-DLA) demonstrated similar relative effects on the four isoenzymes of PDK with the following extent of inhibition: PDK1 > PDK4 ~ PDK2 > PDK3. The inhibition of PDK2, PDK3 and PDK4 was less by R-DLA than R-LA. Oxidized glutathione under similar conditions caused only about 15% reduction in PDK1 activity at 5 mM (results not shown).

The activities of PDKs depicted in Fig. 2 were determined in the oxidized condition of lipoyl moieties of E2 in the presence of NAD$^+$/NADH = 200/1.

TABLE I Activity of PDP1 and PDP2 towards 3 phosphorylation sites of E1 (taken at a saturating concentration) in the presence of R-LA

	Site 1		Site 2		Site 3	
R-LA, mM	PDP1, mU mg^{-1} (%)	PDP2, mU mg^{-1} (%)	PDP1, mU mg^{-1} (%)	PDP2, mU mg^{-1} (%)	PDP1, mU mg^{-1} (%)	PDP2, mU mg^{-1} (%)
0	18.06 (100)	10.15 (100)	75.22 (100)	23.56 (100)	20.82 (100)	13.78 (100)
1	17.94 (99.3)	8.64 (85.2)	72.02 (95.7)	21.75 (93.2)	22.14 (106.3)	13.36 (97.0)
3	16.60 (91.9)	8.61 (84.8)	74.80 (99.4)	18.37 (78.0)	20.52 (99.6)	10.70 (77.7)
5	16.77 (92.9)	6.95 (68.5)	72.85 (96.8)	18.37 (78.0)	20.52 (99.6)	10.33 (75.0)

Activities of PDP1 and PDP2 were determined in the absence or presence of R-LA as indicated by dephosphorylation of E1 (0.33 mg ml^{-1}) individually phosphorylated on site 1, site 2, or site 3 as described in "Materials and Methods Section". PDP activity was calculated from the linear part of the progress curves of the dephosphorylation reactions (8–10 experimental points were used per each calculation). The correlation coefficients were not less than 95%. Similar results were obtained for 2–4 independent experiments.

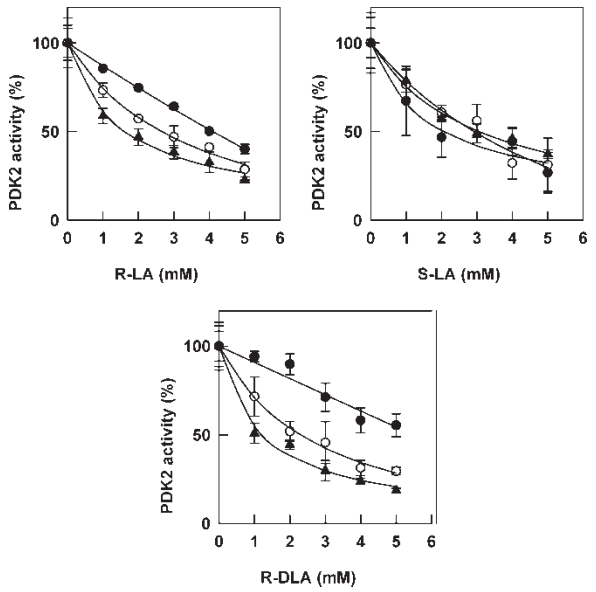

FIGURE 3 Effect of the reduced/acetylated state of lipoyl moieties of E2 on the inhibition of PDK2 by R-LA, S-LA and R-DLA. PDK2 activity was measured in the presence of 0 to 5 mM lipoic acids with reconstituted PDC preincubated for 5 min with $NAD^+/NADH = 796\,\mu M/4\,\mu M$ plus 50 μM CoA (oxidized lipoyl moieties, ●); $NADH/NAD^+ = 600\,\mu M/200\,\mu M$ plus 50 μM CoA (reduced lipoyl moieties, ○); and $NADH/NAD^+ = 600\,\mu M/200\,mM$ plus 50 μM acetyl-CoA (reduced and acetylated lipoyl moieties, ▲). Hundred percent of activity corresponded to: 82 mU mg^{-1}, oxidized lipoyl moieties; 169 mU mg^{-1}, reduced lipoyl moieties: 208 mU mg^{-1}, reduced and acetylated lipoyl moieties. Results are means ± S.D. of 3–4 determinations.

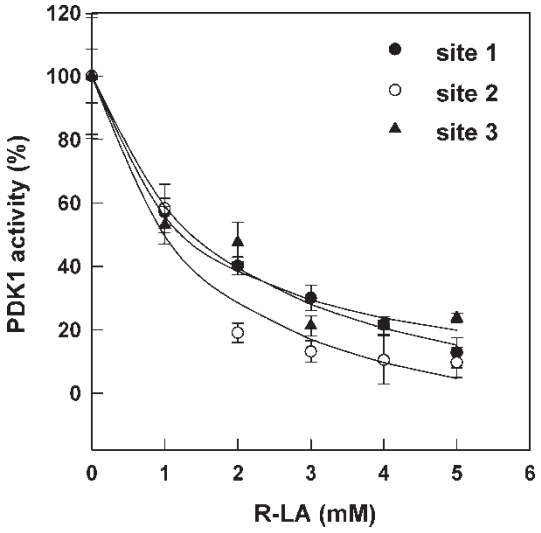

FIGURE 4 R-LA inhibition of PDK1 activity towards the phosphorylation site 1, site 2, and site 3. To determine PDK activity towards sites 1, 2 and 3, PDC was reconstituted using mutant E1s (E1–S2A/S3A for site 1; E1–S1A/S3A for site 2; and E1–S1A/S2A for site 3), E2–BP and E3. Hundred percent of PDK1 activity was: 83 mU mg^{-1} (towards site 1, ●); 7.1 mU mg^{-1} (towards site 2, ○) and 15 mU mg^{-1} (towards site 3, ▲). Results are means ± S.D. of 3–4 determinations.

Activities of PDKs are enhanced by the reduction/acetylation of lipoyl domain of E2.[34] Figure 3 shows how reduction and reduction/acetylation of lipoyl moieties of E2 affected inhibition of PDK2 by R-LA, S-LA, and R-DLA. Only PDK2 displayed differences in the inhibition patterns depending on the redox status of lipoyl groups (results on PDK1, PDK3, PDK4 are not shown). Surprisingly, inhibition of PDK2 by S-LA did not change with the reduction/acetylation of lipoyl groups of E2. The extent of inhibition by R-LA and R-DLA was higher for reduced and reduced/acetylated lipoyl groups compared with oxidized lipoyl groups of E2.

One of the possible explanations of the inhibitory action of lipoic acids on PDK activity was that LA might occupy the active site of E1 (lipoyl moieties are substrates for the reductive acetylation reaction catalyzed by E1) preventing access of PDK to phosphorylation site 1 localized in the substrate channel. Therefore, we investigated how R-LA affected phosphorylation of sites 1, 2, and 3 individually by PDK1. PDK1 is the only isoenzyme that phosphorylated all three sites.[33] Figure 4 shows that R-LA decreased similarly the activities of PDK1 for site 1, site 2, or site 3. Sites 1, 2 and 3 are localized differently in E1 and hence can not be affected in the same way by R-LA. Hence binding of lipoic acids in the active site of E1 can not explain inhibition of E1 phosphorylation by PDKs.

Inhibition of E1 Phosphorylation by Lipoic Acids

PDKs are bound to the lipoyl domains of E2 or BP (PDK1-3 with E2 and PDK4 with E2 and BP) and these interactions enhance their activities. To examine whether lipoic acids could interfere with PDK binding to E2 or BP, we investigated the effect of lipoic acids (0–5 mM) on the PDKs activity towards free E1. Figure 5 compares the effects of lipoic acids (3 mM) on the activities of PDKs towards E1 with activities towards E1 reconstituted in PDC. Both R-LA and S-LA inhibited activities of PDKs (except PDK3) for E1 alone. S-LA demonstrated higher inhibition of PDK1, PDK2 and PDK4 activities for both E1 alone and PDC-E1. Inhibition by R-LA and S-LA was higher for PDK1 and PDK4 than for PDK2 and PDK3 with E1 alone and also for PDC-E1. R-DLA was less effective inhibitor of PDK1, PDK2, PDK4 with phosphorylation of E1 alone than with phosphorylation of PDC-E1. Inhibition of E1 phosphorylation by PDKs in the absence of E2 by lipoic acids indicates that dissociation of PDK from E2 in the presence of lipoic acids could not be an explanation of inhibition of PDC-E1 phosphorylation by PDKs.

Inhibition of PDK1 by R-LA in the Presence of Pyruvate and ADP

To test the effect of lipoic acids on PDKs, the concentration of E1 had to be reduced to the range of

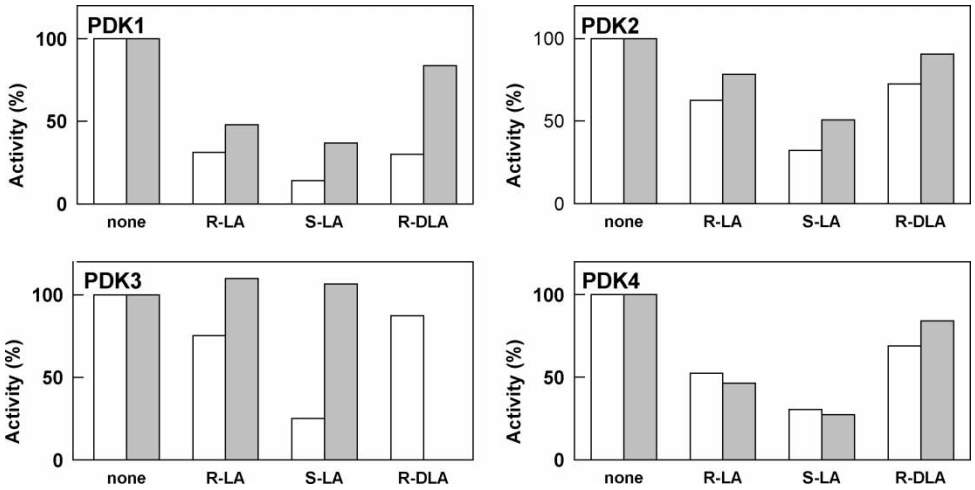

FIGURE 5 Comparisons of the inhibitory effects of lipoic acids on activities of PDK isoenzymes towards PDC (open columns) and E1 alone (gray columns). Activities of PDKs were determined in the presence of 3 mM of lipoic acids. Hundred percent of PDK activities towards E1 alone corresponded to: 31 mU mg^{-1} for PDK1; 27 mU mg^{-1} for PDK2; 5.9 mU mg^{-1} for PDK3; and 32 mU mg^{-1} for PDK4. Hundred percent of PDK activities towards reconstituted PDC corresponded to: 56 mU mg^{-1} for PDK1; 87 mU mg^{-1} for PDK2; 64 mU mg^{-1} for PDK3; and 38 mU mg^{-1} for PDK4. Results are means ± S.D. of 3–4 determinations.

$S_{0.5}$ values for PDKs.[33] To investigate the inhibitory effect further we have determined kinetic parameters of PDK1 for E1 in the presence of R-LA. In the presence of R-LA, V_{max} of PDK1 towards E1 did not change, whereas $S_{0.5}$ increased (from 0.211 μM in the absence of R-LA to 0.422 μM at 1 mM R-LA; 0.685 μM at 2 mM R-LA; 1.037 μM at 3 mM R-LA; and 1.316 μM at 5 mM R-LA), indicating that lipoic acid could be bound on PDK close to the E1-substrate-binding site of PDK.

PDKs are known to be inhibited by ADP, a product of the reaction, and by pyruvate.[36] Pyruvate inhibition is synergistic with ADP. Pyruvate inhibition occurs through its interaction with ADP, as well as preventing ADP dissociation.[36] Figure 6 shows inhibition of PDK isoenzymes with pyruvate when PDK activity was measured towards PDC-E1. Similar to the results reported previously,[22] inhibition was higher for PDK2 > PDK1 > PDK4 > PDK3. Pyruvate was a much better inhibitor than lipoic compounds (10 to 50-fold lower concentrations of pyruvate caused inactivation of PDKs similar to that by R-LA). We have tested the pyruvate analog, pyruvamide, for its inhibitory effect for the first time. Surprisingly, pyruvamide appeared to be a very poor inhibitor of all PDKs, indicating that the regulatory site for pyruvate on PDKs either requires an acidic group for inhibition or the amide group prevents analog binding (Fig. 6).

We have studied inhibition of PDK1 in PDC by various concentrations of R-LA (0–2 mM) in the presence of different concentrations of either pyruvate or ADP as indicated. When ADP was added simultaneously with R-LA, it did not change the level of R-LA inhibition (at 0, 0.2 and 0.6 mM of ADP the level of relative R-LA inhibition was 40.7%, 45.4%, and 49.4%, respectively). However, when pyruvate was added simultaneously with R-LA,

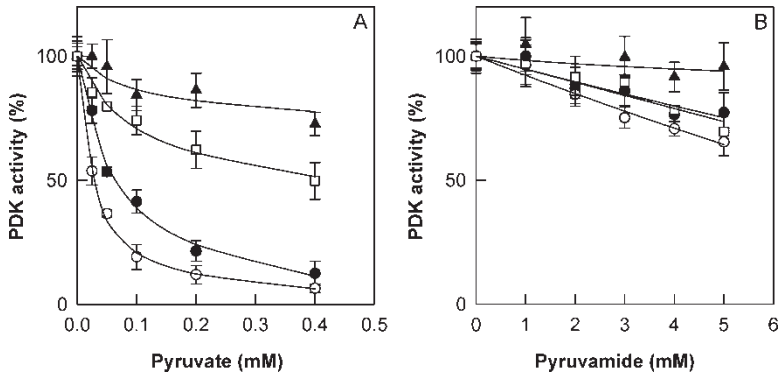

FIGURE 6 Comparison of PDKs inhibition by pyruvate (A) and its analog, pyruvamide (B). Activities of PDKs were determined with reconstituted PDC with oxidized lipoyl moieties of E2 in the presence of pyruvate or pyruvamide. Hundred percent of activities corresponded to: 38 mU mg^{-1} for PDK1 (●); 157 mU mg^{-1} for PDK2 (○); 69 mU mg^{-1} for PDK3 (▲); and 41 mU mg^{-1} for PDK4 (□). Results are means ± S.D. of 3–4 determinations.

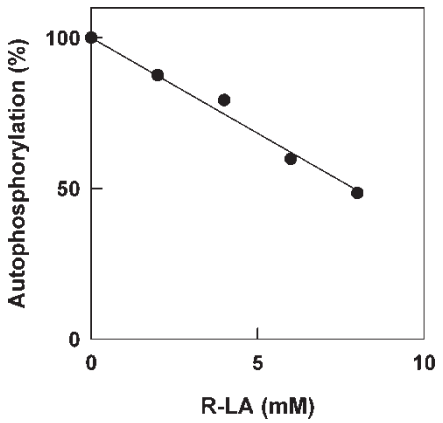

FIGURE 7 Effect of R-LA on autophosphorylation of PDK2. Autophosphorylation of PDK2 was determined in the presence of R-LA by incorporation of ^{32}P from [γ-^{32}P]ATP into PDK2 after a 100-min incubation. Hundred percent of autophosphorylation taken at 100 min corresponded to 1.03 pmole of [γ-^{32}P] per pmol of PDK2.

the inhibition was decreased (at 0, 0.04 and 0.1 mM of pyruvate the level of relative R-LA inhibition was 37.2%, 16.8%, and 13.3%, respectively) (results not shown). The mechanism of inhibition by lipoic compounds is probably different from that of pyruvate. Lipoic compounds did not compete with ADP while pyruvate inhibited PDK synergistically with ADP. On the other hand pyruvate decreased inhibition by lipoic compounds, indicating that lipoic compound binding site and pyruvate binding site affect each other.

Autophosphorylation of PDK2 in the Presence of R-LA

PDKs were found to be autophosphorylated (by incorporation of ^{32}P from [γ-^{32}P]ATP in PDK), however, autophosphorylation was not a part of the catalytic reaction of PDKs.[37] Autophosphorylation of PDK1, PDK3 and PDK4 were only about 20%, 14%, and 2.6%, respectively compared to that of PDK2 (results not shown). The presence of E2 did not alter the level of PDK2 autophosphorylation (results not shown). Because PDK autophosphorylation does not require the presence of other PDC components, it can be used as a test for inhibition of PDK itself. Figure 7 shows that autophosphorylation of PDK2 was decreased in the presence R-LA. This finding indicates that lipoic acids affect PDK2 itself and not E1 or E2.

DISCUSSION

Lipoic Acids Inhibit Phosphorylation of PDC by PDKs

The present study was designed to investigate whether lipoic acid could increase PDC activity by decreasing the extent of phosphorylation of PDC by affecting either PDK isoenzymes or PDP isoenzymes. Surprisingly, instead of expected stimulation of activity of PDPs (as insulin does) the results indicated that lipoic acids did not affect the activity of PDP1 at all and instead inhibited PDP2 to a low extent (Fig. 1 and Table I). These findings eliminated the explanation of the activation of PDC by lipoic acid through the stimulation of PDP. On the other hand, our findings show that both R-LA and R-DLA decreased phosphorylation of PDC by PDK in an isoenzyme-specific manner, and S-LA was a good inhibitor for all four PDKs with similar efficiency (Fig. 2). Inhibition of PDKs was higher than that of PDP2. For instance, 3 mM R-LA caused 69% inhibition of PDK1, 37% of PDK2, 25% of PDK3 and 48% of PDK4, while there was no inhibition of PDP1 and only about 14% inhibition of PDP2.

The Target of Lipoic Acid Inhibition of PDC Phosphorylation

Three components of PDC, namely E1, E2 and PDK, are involved in the phosphorylation reaction. Which one is affected by lipoic compounds? As lipoyl moieties are the substrates for E1, one possible explanation is that inhibition could be due to protection of the active site of E1 by lipoic acid from phosphorylation. However, only phosphorylation site 1 of E1 is in the substrate channel. Sites 2 and 3 are localized outside the substrate channel on the surface of E1.[29,38] Possible binding of lipoic acid in the substrate channel could not have affected PDK1 access to sites 2 and 3. As seen in Fig. 4, inhibition by R-LA was similar for each of the three phosphorylation sites of E1. Compared to the lipoyl moiety of E2, free lipoic acid is a very poor substrate for E1 and could not be bound properly to E1 in the absence of lipoyl domain. Therefore, even site 1 may not be protected from PDKs by lipoic acid.

To determine the target for the inhibitory effect of the lipoic acid, we have excluded E2 by investigating how lipoic acid affected phosphorylation of E1 alone. R-LA and S-LA decreased activities of PDK1, PDK2 and PDK4 towards E1 alone (Fig. 5). Since PDK was suggested to have an SH-group involved in the phosphorylation reaction,[39] oxidation of this group could result in PDK inactivation. If this was the case, then only the oxidized form of lipoic acid would cause inhibition, and inhibition could also be carried out by other oxidized thiols. However, oxidized glutathione did not inactivate PDK to any significant extent, and R-DLA was also an effective inhibitor. The change in the redox status of a PDK SH-group does not appear to explain its inhibition by lipoic acid.

PDKs are related to the eukaryotic serine kinases by catalytic mechanism, but are structurally similar

to bacterial histidine protein kinases.[40] The catalytic mechanism of prokaryotic histidine kinases involves autophosphorylation of a specific histidine residue of the kinases. PDKs are also subjected to autophosphorylation, however, it is not the part of their catalytic mechanism.[37] Autophosphorylation reaction can be performed with only one protein component in PDC, namely PDK. It was found that R-LA decreased the rate of autophosphorylation of PDK2, indicating its interaction with PDK2 (Fig. 7).

Possible Mechanism of PDKs Inactivation by Lipoic Acids

Several inhibitors of PDK were recently tested for the phosphorylation reaction of PDC and synthetic peptide, autophosphorylation of PDK, and for the stimulation of PDC activity in rats, as well as the ability to increase glucose oxidation in rat hepatocytes.[41–43] Several mechanisms of inhibition were suggested involving different sites such as: nucleotide-binding site, pyruvate-binding site and lipoyl domain-binding site in PDK. In the present study, R-LA did not compete with ADP and hence the nucleotide-binding domain of PDK was probably not affected. Pyruvate, in contrast, decreased inhibition by R-LA indicating that R-LA and pyruvate sites could affect each other. Pyruvate was proposed to bind the PDK-ADP intermediate and not directly to the kinase. It was found that inhibition of ADP and pyruvate was synergistic.[36] R-LA could not substitute for pyruvate and hence could not bind at the pyruvate site. Probably the pyruvate site has stringent structural restrictions because pyruvamide, a close analog of pyruvate, is a very poor inhibitor of PDKs (Fig. 6).

Interestingly, the proposed interaction of the lipoyl domain with PDK2 places the lipoyl group of the lipoyl domain close to the active site and nucleotide-binding site.[40] Lipoic acid could affect PDC phosphorylation through several mechanisms. The primary effect appears be on PDK itself. The possible explanation of PDK inhibition by lipoic acid could be that while binding of PDK to the lipoyl domain of E2 can cause a conformational change in PDK resulting in its activation, free lipoic acid could occupy the binding site on PDK of lipoyl moiety of the lipoyl domain of E2. In the absence of the lipoyl domain it would not cause PDK activation and may result in inhibition. We also can not exclude the possibility that interaction of lipoic acid with PDK may interfere with binding of E1 as inhibition was seen only at low E1 concentration.

Possible Impact of Lipoic Acid on the PDC Activity

An increase in glucose metabolism involves at least two critical steps: (i) initially an increase in glucose uptake and (ii) subsequent activation of PDC to enhance oxidation of glucose-derived pyruvate via PDC. Interestingly, R-LA is able to increase the flux at both of these steps. Activity of PDC is regulated mostly by the changes in its phosphorylation status. Expression of PDP2 was shown to be down-regulated during starvation and streptozotocin-induced diabetes.[44] The levels of two isoenzymes of PDKs, PDK4 and PDK2 increase in certain metabolic conditions (diabetes, hyperthyroidism and starvation).[45–48] Our findings show that lipoic acid (at a millimolar concentration) inhibits PDKs. However, the maximal serum concentration observed in humans after 1–3 weeks of daily R-LA administration was in the range of 25–50 μM.[26] The direct inhibition of PDKs by lipoic acid may not be the only mechanism of the PDC activation. Additional inhibition of PDKs could be achieved by pyruvate derived from the enhanced glucose uptake. Our findings provide one of the possible mechanisms by which R-LA increases PDC activity by inhibiting PDKs and hence lowering of the phosphorylation state of this multienzyme complex.

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