

R- α -Lipoic Acid Action on Cell Redox Status, the Insulin Receptor, and Glucose Uptake in 3T3-L1 Adipocytes

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The insulin signaling pathway has been reported to mediate R- α -lipoic acid- (R-LA)-stimulated glucose uptake into 3T3-L1 adipocytes and L6 myotubes. We investigated the role of the thiol antioxidant dihydrolipoic acid (DHLA) and intracellular glutathione (GSH) in R-LA-stimulated glucose transport and explored the hypothesis that R-LA could increase glucose uptake into 3T3-L1 adipocytes in an oxidant-mimetic manner. R-LA pretreatment of 3T3-L1 cells stimulated glucose transport at early time points (30 min–6 h), whereas it inhibited glucose uptake at later time points. Analysis of the oxidized and reduced content of LA in cells and medium showed that >90% of lipoic acid present was in its oxidized form. Furthermore, all oxidized forms of LA (S-, R-, and racemic LA) stimulated glucose uptake, whereas the reduced form, dihydrolipoic acid, was ineffective. Intracellular GSH levels were not changed at the early time points (before 12 h), while longer preincubation (24–48 h) of cells with R-LA significantly increased intracellular GSH. Pretreatment of adipocytes with R-LA increased intracellular peroxide levels at early time points (30 min–6 h), after which it was decreased (12–48 h). R-LA also increased tyrosine phosphorylation of immunoprecipitated insulin receptors from 3T3-L1 adipocytes. These results indicate that (i) 3T3-L1 adipocytes have a low capacity to reduce R-LA and the oxidized form of lipoic acid is responsible for stimulating glucose uptake, (ii) R-LA modulates glucose uptake by changing the intracellular redox status, and (iii) the insulin receptor is a potential cellular target for R-LA action.

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Key Words: α -lipoic acid; intracellular redox state; insulin signaling; glucose transport.

α -Lipoic acid (LA)³ has been used in Germany for the relief of symptoms of diabetic neuropathy (1, 2). A growing body of evidence suggests that LA may have potential therapeutic value in lowering elevated glucose levels in diabetic conditions. Oral or intravenous administration of LA was shown to increase insulin sensitivity in individuals with type 2 diabetes (3–5). In animal models of diabetes, chronic LA administration increased GLUT-4 protein level in muscle membranes, improved insulin-stimulated 2-deoxyglucose uptake into isolated skeletal muscles, and reduced blood glucose level indicating that LA might enhance the capacity of insulin stimulated glucose transport and utilization in skeletal muscle (6–8). However, LA itself was also shown to enhance glucose uptake into epitrochlearis muscles isolated from both obese (*fa/fa*) and lean (*fa/-*) Zucker rats (9).

Mechanistic studies conducted in insulin responsive cells in culture demonstrated that LA rapidly stimulates glucose uptake by activating elements of the insulin signaling pathway (10, 11). Insulin action is initiated by insulin binding to the extracellular α -subunit of the insulin receptor (IR), which transmits a transmembrane signal activating the intracellular tyrosine kinase domain of the β -subunits (12, 13). Following IR autophosphorylation, the tyrosine kinase domain of the β -subunit tyrosine phosphorylates a number of cel-

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³ Abbreviations used: DCF, dichlorofluorescein; DHLA, dihydrolipoic acid; GSH, reduced glutathione; IR, insulin receptor; LA, α -lipoic acid; PI 3-kinase, type I phosphatidylinositol 3-kinase; TBH, tert-butyl hydroperoxide.

lular proteins, including members of the insulin receptor substrate (IRS1/2/3/4) family (14). Engagement of the SH2 domains in the p85 regulatory subunit of type I phosphatidylinositol 3-kinase (PI 3-kinase) with tyrosine phosphorylated IRS serves to activate the catalytic p110 subunit and to target the PI 3-kinase to its downstream effectors such as protein kinase B/Akt (15, 16). Activation of PI 3-kinase was shown to be necessary for insulin-stimulated translocation of glucose transporter-4 (GLUT4) from a sequestered intracellular pool to the plasma membrane (17, 18). Using L6 myotubes and 3T3-L1 adipocytes as a model of muscle and fat cells in culture, *R*-LA was shown to increase tyrosine phosphorylation of IR and IRS-1, to enhance PI 3-kinase and Akt1 activities, to elevate GLUT4 content in the plasma membranes, and to increase glucose uptake into the cells (19, 20). Although these findings have substantially increased our understanding of LA action on the insulin signaling pathway, the basis for the ability of *R*-LA to activate the elements of this pathway, its mode of action and molecular target(s) are not yet known.

Intracellular redox status is known to play an important role in the modulation of insulin action. Treatment of IR-transfected Chinese hamster ovary cells with antioxidants such as *N*-acetyl-cysteine or butylated hydroxyanisole inhibited insulin responsiveness, whereas partial inhibition of glutathione metabolism, which intracellularly induces a mild oxidative stress condition, stimulated IR tyrosine phosphorylation when measured *in vitro* (21). A similar increase in IR kinase activity was observed following cell treatment with hydrogen peroxide (22–25). Moreover, it has been found that oxidation of critical cysteine residues in the IR β -subunit may also result in an increase in its intrinsic tyrosine kinase activity, whereas low concentrations of dithiothreitol inactivates the IR kinase, supporting the importance of oxidation of critical thiol groups in activation of the insulin signaling pathway (26, 27). LA is known to be intracellularly reduced to the potent dithiol antioxidant, dihydrolipoic acid (DHLA), and to enhance intracellular reduced glutathione (GSH) levels in a variety of cells (28, 29). Based on its chemical structure, LA, an eight-carbon compound that contains a disulfide bond can also act as a mild oxidant molecule before its conversion to the reduced form. This study was undertaken to explore the hypothesis that *R*-LA increases glucose uptake into 3T3-L1 adipocytes by an oxidant-mimetic manner.

MATERIALS AND METHODS

Chemicals. Glucose, dihydrolipoic acid, cytochalasin B, *tert*-butyl hydroperoxide, wortmannin, insulin, 3-isobutyl-1-methylxanthine, 2',7'-dichlorodihydrofluorescein diacetate, and dexamethasone were purchased from Sigma Chemical Co. (St. Louis, MO). 2-[¹⁴C(U)]-deoxy-D-glucose and [γ -³²P]ATP was obtained from NEN Life Science

Products (Boston, MA). Protein A/G agarose was from Calbiochem (San Diego, CA). Monoclonal anti-insulin receptor beta chain antibody (Insulin R β , 29B4) was from Santa Cruz Biotech Inc. (Santa Cruz, CA). 4G10 anti-phosphotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY). Hybond ECL nitrocellulose membrane was from Amersham (Arlington Heights, IL). Cell culture reagents and other chemicals were purchased from Life Technologies (Gaithersburg, MD).

Cell culture. 3T3-L1 preadipocytes (American Type Culture Collection) were grown to confluence in Dulbecco's modified Eagle medium (DMEM) containing 10% FCS, 100 units/ml of penicillin, and 100 μ g/ml of streptomycin as previously described (30). Cells were induced to differentiate to adipocytes 48 h after confluence by changing the medium to complete DMEM supplemented with 5 μ g/ml of insulin, 0.25 μ M of dexamethasone, and 0.5 mM of 3-isobutyl-1-methylxanthine for 72 h. Cells were then maintained in DMEM containing 10% FCS and antibiotics and used 8–10 days after differentiation when more than 90% of cells exhibited the adipocyte phenotype.

2-Deoxyglucose uptake. Uptake of 2-deoxyglucose by 3T3-L1 adipocytes was measured as previously described (31). Briefly, cells were washed twice with phosphate-buffered saline containing 2.5 mM MgCl₂, 1 mM CaCl₂, and 20 mM Hepes, pH 7.4, and incubated with test compounds in the same buffer at 37°C. Transport assay was then initiated by addition of 2-[¹⁴C(U)]-deoxy-D-glucose (25 μ M; 10 μ Ci/ml) to each well for 10 min at 37°C. In some experiments, glucose transport was measured after addition of a mixture of 2-[¹⁴C(U)]-deoxy-D-glucose (10 μ M; 2 μ Ci/ml) and D-glucose (5 mM). Transport assay was terminated by addition and subsequent washing of cells with ice-cold PBS. Cells were lysed in 10% SDS or 50 mM NaOH. The radioactivity was determined by scintillation counting of lysates extracted in SDS, whereas total protein was determined in lysates extracted in NaOH using the Bradford procedure (Bio-Rad Laboratories, Richmond, CA). Glucose uptake values were corrected for non-carrier-mediated transport by measuring glucose uptake in the presence of 10 μ M cytochalasin B.

Intracellular reactive oxygen species. Intracellular oxidants were detected using 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) (32). Briefly, cells seeded in 12-well plates were incubated for 2–48 h with *R*-LA (250 μ M) and loaded with H₂DCF-DA (20 μ M) for the last 30 min in PBS containing *R*-LA. For some experiments cells were washed and incubated with *R*-LA (0.25–1 mM) and H₂DCF-DA (20 μ M) for 30 min. Following the treatments, cells were gently scraped by a lifter and suspended in the same media. For detection of intracellular fluorescence, cells were excited using a 488-nm argon-ion laser in a flow cytometer (XL, Coulter, FL). The dichlorofluorescein (DCF) emission was recorded at 530 nm. Data were collected from at least 20,000 cells.

Intracellular glutathione. Cells seeded in 12-well plates were incubated with 250 μ M of *R*-LA for various times. Cells were then washed with ice-cold PBS, treated with 4% (w/v) *m*-phosphoric acid and scraped. All samples were immediately frozen in liquid nitrogen and stored at –70°C until analysis by HPLC. Immediately before the assay, samples were thawed, vortexed, and then centrifuged at 15,000g for 2 min at 4°C. The clear supernatant was removed and injected into the HPLC system. HPLC-EC detection of intracellular GSH was performed using an ESA coulometric detector (Chelmsford, MA) as described previously (33).

Quantitation of α -lipoic acid and dihydrolipoic acid. Cells seeded in 12-well plate were incubated with 250 μ M of *R*-LA for various times. The media were collected. Cells were scraped from the wells and collected in 4% *m*-phosphoric acid (w/v). The level of racemic LA and DHLA in the cell lysate and cell culture media were determined by HPLC using ESA coulometric detector (Chelmsford) as described (34).

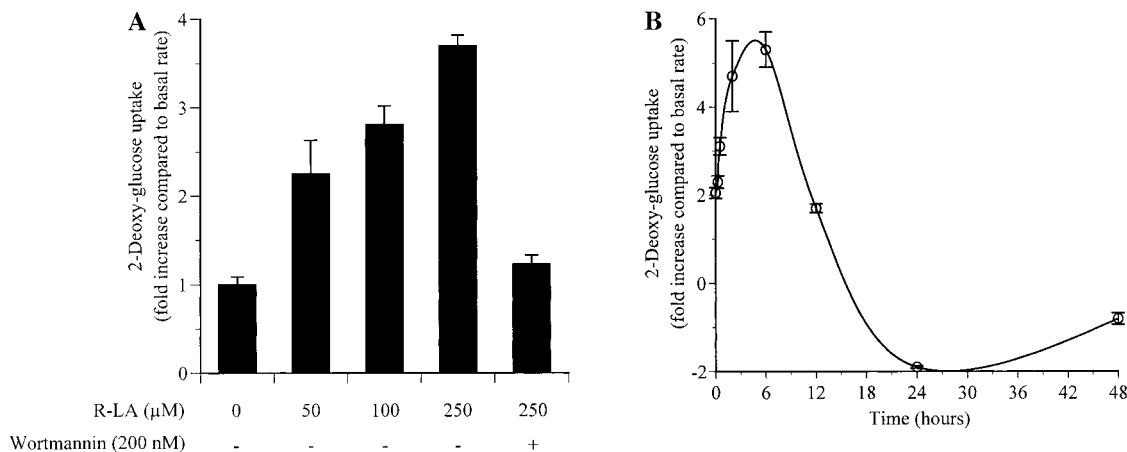


FIG. 1. Effect of R-LA on 2-deoxyglucose uptake in 3T3-L1 adipocytes. (A) Concentration dependence of R-LA action. Cells were preincubated with R-LA at indicated concentrations for 30 min before glucose transport assay. When present, wortmannin was added to cells 30 min prior to R-LA preincubation. (B) Time dependence of R-LA action. Cells were preincubated with 250 μ M of R-LA for indicated times before glucose transport assay. Glucose uptake values were corrected for non-carrier-mediated transport by measuring glucose uptake in presence of 10 μ M cytochalasin B added just before start of transport assay and were expressed as fold increase compared to the basal rate.

Cell lysis and IR immunoprecipitation. Cells in T₇₅ flasks were serum deprived for 5 h, washed three times with ice-cold PBS, and lysed in lysis buffer (50 mM Tris-HCl buffer, pH 7.2, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 25 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, 5 μ g/ml leupeptin, and 2 μ g/ml aprotinin). After centrifugation, the clear supernatant was incubated with 2 μ g/ml of monoclonal anti-insulin receptor beta chain antibody (Insulin R β , 29B4) overnight at 4°C. The immune complexes were collected by incubating the sample with protein A plus protein G agarose for overnight at 4°C. The immunoprecipitated insulin receptors were washed three times with lysis buffer and twice with phosphorylation buffer (20 mM Hepes, pH 7.2, 100 mM NaCl, 5 mM MgCl₂, 5 mM MnCl₂, and 100 μ M sodium orthovanadate).

In vitro kinase assay and detection of IR β -subunit phosphorylation. *In vitro* kinase assay was performed as previously described (21). Briefly, aliquots of immunoprecipitated insulin receptors were incubated, in the phosphorylation buffer, with 100 nM insulin or 250 μ M R- or S-LA for 30 min at RT, and *in vitro* phosphorylation was conducted for 15 min at 30°C after adding 6.5 μ Ci [γ -³²P]ATP. The reaction was stopped by boiling the samples in Laemmli sample buffer and proteins were separated by reducing SDS-PAGE. ³²P incorporation was then determined by autoradiography. To detect tyrosine phosphorylation of insulin receptor beta domain, the *in vitro* phosphorylation was performed by adding 1 mM cold ATP for 20 min at 30°C. The proteins were separated electrophoretically and transferred to Hybond ECL nitrocellulose membrane. The membrane was blocked in Blocker Casein in PBS (Pierce, IL), immunoblotted with monoclonal anti-phosphotyrosine antibody (4G10), and tyrosine phosphorylated β -subunit was detected using an ECL^{plus} kit (Amersham, NJ).

Preparation of α -lipoic acid isomers and dihydrolipoic acid. 50 mM stock solutions of R-LA, S-LA, and racemic LA were prepared in 20 mM Hepes, pH 7.4. A 250 mM stock solution of DHLA was prepared in DMSO. A matched volume of DMSO was added to the control cells when it was used as a solvent.

Data presentation. Data are reported as mean \pm SD of at least three independent experiments performed in triplicate. Comparison between multiple groups was made by analysis of variance (ANOVA) or two-tailed z test. The minimum level of significance was set at $P < 0.05$.

RESULTS

Effect of R- α -lipoic Acid on Glucose Transport

In 3T3-L1 adipocytes basal glucose transport was 1.46 ± 0.19 pmol/min/mg protein. Insulin (100 nM) stimulation increased glucose uptake into the cells by 4.6-fold (6.7 ± 0.30 pmol/min/mg protein). Pretreatment of 3T3-L1 adipocytes with R-LA for 30 min increased glucose transport dose dependently relative to the control cells at concentrations between 50–250 μ M (Fig. 1A). Wortmannin, a PI-3-kinase inhibitor, inhibited R-LA-stimulated glucose uptake. Pretreatment with R-LA over 48 h exhibited a biphasic effect on glucose uptake into 3T3-L1 adipocytes (Fig. 1B). Addition of R-LA just before the start of the transport assay (0 time) increased glucose uptake by twofold. R-LA pretreatment increased glucose transport with a maximal 5.3-fold over basal rate obtained by 6 h preincubation. However, longer preincubation (24 or 48 h) inhibited glucose uptake into 3T3-L1 adipocytes.

Reduction of R-LA by 3T3-L1 Adipocytes

The capacity of adipocytes to reduce R-LA and thereby to generate the strong antioxidant, DHLA, was evaluated following incubation with 250 μ M of R-LA for various times (Table I). Following rapid extraction and snap injection into an HPLC-EC system 94.4% of added R-LA was found to be remain in the oxidized form after 6 h preincubation. The total amount of DHLA detected in cells and their supernatants at this time point was approximately 5.0% of the added R-LA. Lower amounts of both LA and DHLA was found in the cells and their supernatants after 48 h incubation with R-LA.

TABLE I
Reduction of R-LA by 3T3-L1 Adipocytes

	6 h		48 h	
	α -Lipoic acid	Dihydrolipoic acid	α -Lipoic acid	Dihydrolipoic acid
Supernatant (% of added LA)	94.4 \pm 13.5	4.9 \pm 0.91	33.5 \pm 4.0	1.4 \pm 0.55
Cells (% of added LA)	0.56 \pm 0.19	0.005 \pm 0.003	0.3 \pm 0.07	0.0005 \pm 0.001

Note. Cells were incubated with 250 μ M of R-LA for 6 or 48 h. The level of racemic LA and DHLA in cell lysates and supernatants was measured by HPLC and expressed as percentage of total added R-LA.

Effect of Stereoisomers of Lipoic Acid and Dihydrolipoic Acid on Glucose Transport

The effect of oxidized stereoisomers of α -lipoic acid, *R*- and *S*-LA, their racemate mixture, racemic LA, and the reduced racemate form, DHLA, on glucose uptake was examined after 30 min preincubation of cells with 250 μ M of the either test compounds (Fig. 2). *R*-, *S*-, and racemic LA increased glucose transport by 4.1 \pm 1.6-, 3.1 \pm 1.3-, and 5.0 \pm 0.9-fold, respectively, over the basal rate. A significantly higher increase in glucose transport was obtained by racemic LA pretreatment compared to *S*-LA ($P < 0.01$). DHLA increased glucose uptake by 1.38 \pm 0.1-fold over the basal rate. DHLA-induced glucose transport was significantly less than that elicited by the oxidized stereoisomers, *R*- and *S*-LA, and racemic LA ($P < 0.001$).

Effect of *R*- α -lipoic Acid on Intracellular GSH Content

The intracellular GSH content of 3T3-L1 adipocytes was 3.2 \pm 0.20 nmol/mg protein. A statistically nonsig-

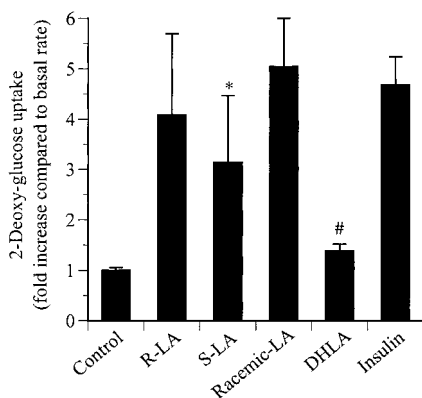


FIG. 2. Comparative effect of α -lipoic acid stereoisomers and DHLA on glucose transport. 3T3-L1 adipocytes were incubated with 250 μ M of R-LA, S-LA, racemic LA, or DHLA for 30 min before glucose transport assay. Insulin (100 nM) was added just before the start of glucose transport assay. The number of independent experiments performed in triplicate for R-LA, S-LA, racemic-LA, DHLA, and insulin-treated cells were 6, 6, 5, 3, and 3, respectively. *Significantly different from racemic LA, $P < 0.01$ (ANOVA). #Significantly different from S-, R-, and racemic LA, $P < 0.001$ (ANOVA).

nificant elevation in intracellular GSH level was observed after 12 h incubation of cells with 250 μ M of *R*-LA (Fig. 3). However, preincubation of cells with *R*-LA for 24 or 48 h significantly increased, as compared to the basal, the intracellular level of GSH to 7.0 \pm 0.47 and 9.5 \pm 2.41 nmol/mg protein, respectively ($P < 0.05$). No change was detected in intracellular GSH levels after incubation with *R*-LA (250 μ M) for 0.5–2 h (not shown).

Tert-Butyl Hydroperoxide (TBH) Acutely Stimulates Glucose Transport

Pretreatment with the strong oxidant TBH for 30 min dose dependently increased glucose uptake into 3T3-L1 adipocytes (Fig. 4). Similar to *R*-LA, the TBH-stimulated glucose uptake was completely abrogated with wortmannin, the PI 3-kinase inhibitor.

R- α -lipoic Acid Increases Intracellular Oxidant Levels

Intracellular DCF fluorescence was determined after incubation of 3T3-L1 adipocytes with *R*-LA at various concentrations and time points. *R*-LA pretreatment

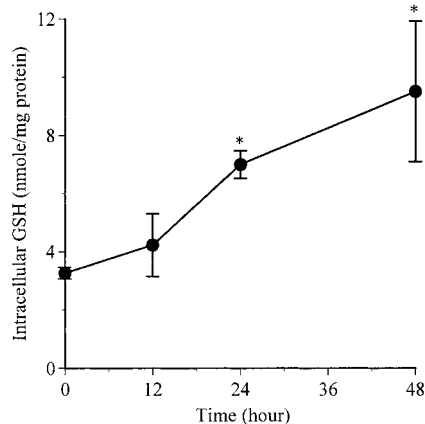


FIG. 3. Effect of *R*-LA on intracellular GSH content. 3T3-L1 adipocytes were incubated with 250 μ M of *R*-LA for indicated times and intracellular GSH was measured by HPLC as described under Materials and Methods. *Significantly different from 0 time, $P < 0.05$ (ANOVA).

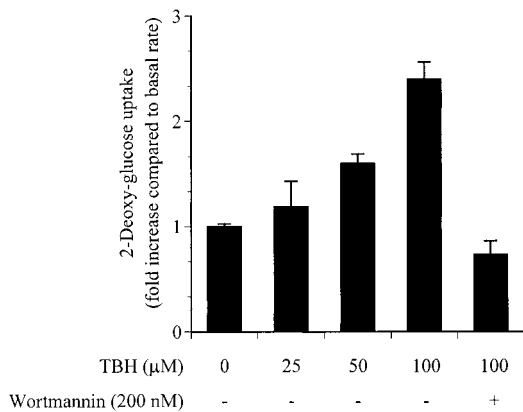


FIG. 4. TBH increases glucose uptake into 3T3-L1 adipocytes. Cells were preincubated with indicated concentrations of TBH in the presence and absence of wortmannin (200 nM) for 30 min at 37°C before glucose transport assay.

increased H₂DCF oxidation as estimated by the elevated DCF fluorescence (Fig. 5A). To evaluate whether the increase in intracellular oxidant level by *R*-LA is superimposed with the temporal kinetics of glucose uptake, a second experiment was performed in which cells were incubated with 250 μM *R*-LA over 48 h. The DCF signal was at a maximum following a 6-h preincubation of cells with *R*-LA (Fig. 5B).

R-α-lipoic Acid Facilitates Insulin Receptor Autophosphorylation

The direct effect of *R*- and *S*-LA on insulin receptor activation was analyzed by measuring its kinase activity *in vitro*. Preincubation of immunoprecipitated IR with insulin for 30 min strongly increased autophosphorylation of the IR β-subunit (Fig. 6). *R*-LA (250 μM) pretreatment of immunoprecipitated IR for 30 min in-

creased ³²P incorporation into and tyrosine phosphorylation of IR β-subunit to a comparable degree as insulin-treated immunoprecipitated IR. However, a relatively weaker phosphotyrosine signal, as compared to insulin or *R*-LA, was observed after treatment of immunoprecipitated IR with *S*-LA.

DISCUSSION

α-Lipoic acid has been the focus of intensive research in the last few years (28, 29). The oxidized form is reduced by the multienzyme α-keto acid dehydrogenase complexes resulting in formation of the potent dithiol antioxidant, DHLA (35). The oxidized form has also been proposed to function as an antioxidant due to its ability to react with number of reactive oxygen species *in vitro* (28, 29). However, our results point to the fact that the oxidized form of LA activates the insulin signal transduction pathway by acting as a pro-oxidant.

3T3-L1 Adipocytes Possess a Low Capacity to Reduce R-α-lipoic Acid and the Oxidized Isoforms Are Effective in Stimulating Glucose Transport

Pretreatment of cells with *R*-LA for times as brief as 10 min was found to stimulate glucose transport into 3T3-L1 adipocytes. In agreement with Klip *et al.* (11, 19), wortmannin, a relatively specific inhibitor of PI 3-kinase, abrogated the ability of *R*-LA to stimulate glucose transport indicating that PI 3-kinase mediates the stimulation of glucose transport by *R*-LA. However, in spite of the reported millimolar amounts used in these studies (11, 19), our investigations show that the concentration of *R*-LA required to stimulate glucose transport is in the micromolar range, which is comparable with its plasma level after intravenous or oral

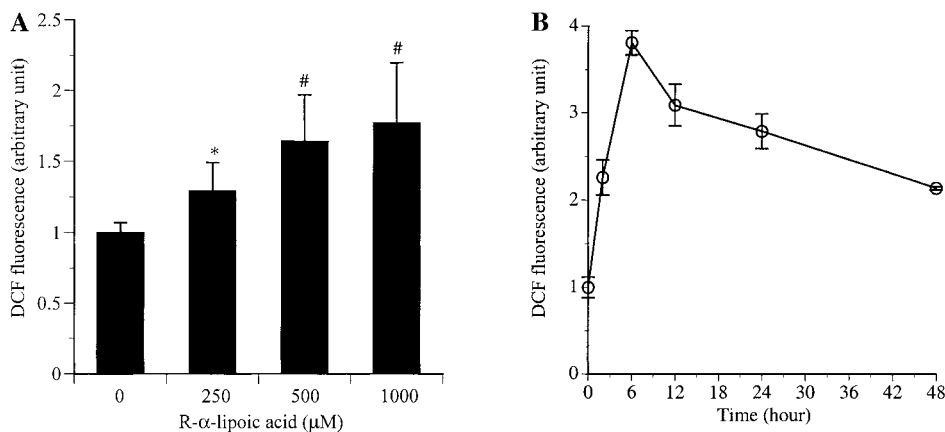


FIG. 5. Effect of *R*-LA on intracellular level of oxidants in 3T3-L1 adipocytes. Cells were preincubated with indicated concentrations of *R*-LA for 30 min (A) or with 250 μM *R*-LA for various times (B). Flow cytometric measurement of DCF fluorescence was determined as described in Materials and Methods. y-Axes represent DCF fluorescence emission recorded in a 4-decade log scale. **P* < 0.05 and #*P* < 0.01 compared with control (*z* test, two-tailed).

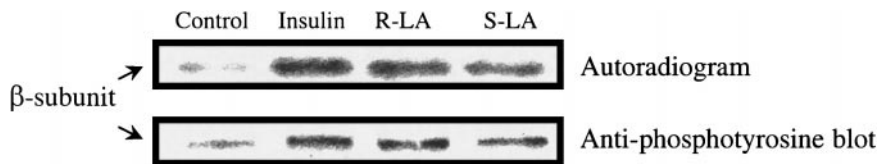


FIG. 6. R- and S-LA facilitate insulin receptor autophosphorylation *in vitro*. Immunoprecipitated IR was incubated with 250 μ M of R- or S-LA or with 100 nM of insulin for 30 min and then subjected to *in vitro* kinase assay in the presence of either ATP or γ - 32 P-ATP. Proteins were separated by SDS-PAGE. 32 P incorporation was determined by autoradiography (upper panel). Tyrosine phosphorylation was analyzed with the monoclonal phosphotyrosine antibody 4G10 by ECL (lower panel). A representative blot of three independent experiments is shown.

administration of 200–600 mg of LA (36, 37). R-LA was shown to be more potent than the S isoform in its ability to improve insulin-stimulated glucose uptake in skeletal muscles from obese Zucker rats or to stimulate glucose transport in L6 myotubes and 3T3-L1 adipocytes (7, 11, 19). In the present study, although S-LA-stimulated glucose uptake was lower than that elicited by R-LA, it did not reach significance, whereas racemic LA was found significantly more effective than S-LA.

Analysis of cellular and extracellular levels of LA and DHLA showed that after 6 h preincubation the majority (>90%) of LA was present in the oxidized form. Lower amounts of LA and DHLA in both cells and their supernatants were detected after 48 h incubation with R-LA, which might be due to cellular metabolism. Furthermore, when added exogenously, DHLA was far less effective in stimulating glucose transport than the oxidized isoforms. A slight increase in glucose transport observed upon stimulation with DHLA might likely be due to the LA generated from the spontaneous oxidation of DHLA in the aqueous medium. Taken together, these findings show that the oxidized isoforms, R- and S-LA, or their racemic mixtures, but not the reduced form, are effective in stimulating glucose uptake into 3T3-L1 adipocytes.

R- α -lipoic Acid Modulates Glucose Transport by Changing Intracellular Redox Status

Intracellular GSH level was unchanged by preincubation of 3T3-L1 adipocytes with R-LA for up to 12 h, demonstrating that alterations in GSH levels are not involved in R-LA-stimulated glucose uptake observed at earlier time points. The fact that DHLA is not also implicated in R-LA action and only the oxidized isoforms are effective in stimulating glucose transport implies that R-LA, an eight-carbon compound with a functional disulfide group in the dithiolane ring, likely acts as a pro-oxidant. The pro-oxidant effect of LA was clearly demonstrated using H₂DCF-DA, which is an indicator of intracellular oxidant levels (38–40). DCF fluorescence measurements showed that treatment with R-LA increased intracellular oxidant levels, which reached their maximal level at 6 h. TBH, a well-known oxidant, also increased DCF fluorescence

(data not shown) and stimulated glucose transport in a wortmannin sensitive manner, indicating that the insulin signaling pathway is oxidant sensitive. Indeed, hydrogen peroxide was hypothesized to act as a second messenger for the observed effects of insulin (41). Recently, human fat cells were shown to possess a multireceptor-linked, plasma membrane-bound H₂O₂ generating system that is under antagonistic control by a large and diverse group of hormones, including insulin, the β -adrenergic agonist isoproterenol, and different isoforms of fibroblast and platelet-derived growth factors, providing further evidence in support of a second messenger function for oxidants such as hydrogen peroxide (42–46).

The maximal intracellular level of oxidants was observed after 6 h pretreatment of 3T3-L1 with R-LA, a finding that is consistent with the kinetics of the R-LA-stimulated elevations of glucose transport. However, longer incubation (>12 h) of cells with R-LA significantly increased cellular GSH levels while at the same time points the intracellular level of oxidants and glucose transport rate were decreased. Hence, a parallel behavior was observed between the intracellular oxidant level, GSH level, and R-LA-stimulated glucose uptake, suggesting that at the early time points (up to 6 h) R-LA likely increases glucose transport by changing the intracellular redox status toward an oxidizing condition whereas at later time points R-LA, by increasing the intracellular GSH content, shifts the intracellular environment toward more reducing conditions, which lowers the rate of glucose transport.

It should be emphasized that whether the observed elevation in GSH level upon treatment with R-LA is due to an improvement in the utilization of cysteine (47), an adaptive response, or is mediated by another mechanism warrants further investigation. However, the amount of R-LA reduced to DHLA seems to be adequate to reflect the changes in the redox status of sensitive cellular proteins when cell volume is taken into account. Given the fact that mammalian cells are 10–30 μ m in diameter (corresponding to an average inner volume of approximately 7×10^{-9} ml), the total concentration of DHLA formed under our experimental

condition by reduction of 4.9% of the added *R*-LA was calculated as 5.0 mM.

Insulin Receptor as a Target of R-LA Action

The mechanism by which *R*-LA and oxidants such as hydrogen peroxide or pervanadate exert insulin-like effects in cells is not known. Although this effect might partly be due to inhibition of protein tyrosine phosphatases, which are sensitive to inactivation by oxidants (48, 49), several lines of evidence suggests that the functional activity of the insulin receptor can be also modulated by alteration of redox state of the cysteine residues present in IR α - and β -subunits (26, 27, 50). When incubated with immunoprecipitated IR, *R*-LA increased tyrosine phosphorylation of IR, indicating that *R*-LA facilitates IR autophosphorylation. Consistent with the stimulation of glucose transport, *S*-LA was found to be less effective in facilitating IR autophosphorylation. The basis for relatively lower efficacy of *S*-LA observed in this and previous studies (7, 11, 19) is not known. Since sodium orthovanadate, a potent inhibitor of protein tyrosine phosphatases, was present in the phosphorylation buffer, the observed effect of LA on IR autophosphorylation is not due to the oxidative inactivation of protein tyrosine phosphatases, which might be brought down during the immunoprecipitation of IR. However, whether the observed effect of *R*-LA on IR autophosphorylation is due to oxidation of the cysteine residues present in the β -subunit and whether disulfides other than LA are able to facilitate IR autophosphorylation remain to be elucidated.

Taking into consideration that pharmacokinetic studies have shown a relatively short half-life (30 min) for LA (36), our results favor the hypothesis that *R*-LA may activate the insulin signaling pathway in insulin responsive tissues, which possess a low capacity to reduce LA such as adipose tissue by increasing intracellular oxidant levels and/or directly activating IR.

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