Protection Against Oxidative Stress–Induced Insulin Resistance in Rat L6 Muscle Cells by Micromolar Concentrations of α-Lipoic Acid

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In diabetic patients, α-lipoic acid (LA) improves skeletal muscle glucose transport, resulting in increased glucose disposal; however, the molecular mechanism of action of LA is presently unknown. We studied the effects of LA on basal and insulin-stimulated glucose transport in cultured rat L6 muscle cells that overexpress GLUT4. When 2-deoxy-D-glucose uptake was measured in these cells, they were more sensitive and responsive to insulin than wild-type L6 cells. LA, at concentrations ≤1 mmol/l, had only small effects on glucose transport in cells not exposed to oxidative stress. When cells were exposed to glucose oxidase and glucose to generate H_2O_2 and cause oxidative stress, there was a marked decrease in insulin-stimulated glucose transport. Pretreatment with LA over the concentration range of 10–1,000 μmol/l protected the insulin effect from inhibition by H_2O_2. Both the R and S isomers of LA were equally effective. In addition, oxidative stress caused a significant decrease (~50%) in reduced glutathione concentration, along with the rapid activation of the stress-sensitive p38 mitogen-activated protein kinase. Pretreatment with LA prevented both of these events, coincident with protecting insulin action. These studies indicate that in muscle, the major site of insulin-stimulated glucose disposal, one important effect of LA on the insulin-signaling cascade is to protect cells from oxidative stress–induced insulin resistance. *Diabetes* 50:404–410, 2001

α-Lipoic acid (1,2-dithiolane-3-pentanoic acid; LA), an analogue of octanoic acid, is present in food and is synthesized by the liver and other tissues. LA is a natural cofactor in the pyruvate dehydrogenase complex where it binds acyl groups and transfers them from one part of the complex to another (1). LA is also a potent antioxidant. Three distinct antioxidant actions of LA and its reduced form, dihydrolipoic acid, have been observed: reactive oxygen species scavenging activity; capacity to regenerate endogenous antioxidants, such as glutathione and vitamins C and E; and metal-chelating activity (1,2). Oxidative stress originating from improper control of the reduction of O_2 is believed to play a role in the tissue and cellular damage caused by a variety of conditions, including diabetes, neurodegenerative disease, infection, aging, and ionizing radiation (3,4).

LA levels are decreased in diabetic patients (5). In Germany, LA has been used for 30 years to treat diabetic neuropathy and liver cirrhosis (2,3,6). It was believed that both the oxidative stress and abnormal 2-oxo-acid oxidation that occur in these conditions are corrected by LA administration. LA has also been used to treat heavy metal poisoning via its metal chelating activity (1).

Evidence suggests that improvements in glucose metabolism occur in diabetic animals and diabetic humans treated with LA (7). It was first reported that LA stimulates glucose utilization in rat hemidiaphragm studied in vitro (8). More recent studies have indicated that LA administration to obese Zucker (*fa/fa*) rats improves insulin-stimulated glucose uptake in muscle (9–11). In addition, in diabetic and nondiabetic fasted rats, LA was reported to cause acute hypoglycemia by decreasing hepatic glucose output (12). This effect on hepatic glucose output could have been attributable to an effect of LA on the liver directly or on adipose tissue, where free fatty acid release regulates hepatic glucose output (13). In spontaneously hypertensive rats, dietary supplementation with LA lowered systolic blood pressure, glucose and insulin levels, and tissue aldehyde conjugates, and attenuated adverse renal vascular changes (14).

In patients with insulin-resistant type 2 diabetes, chronic and acute parenteral administration of LA improves insulin-mediated glucose disposal by 30 and 55%, respectively (15,16). More recently, it has been observed that chronic oral administration of LA exerts a small but significant effect on insulin sensitivity in patients with type 2 diabetes (17,18). Because the major action of insulin in vivo is to enhance glucose disposal via skeletal muscle glucose transport, it is likely that skeletal muscle is a primary target for LA action.

LA has been studied in cultured cells in order to understand and characterize its effects on glucose metabolism. In 3T3-L1 adipocytes, one group reported that oxidative stress induced by the generation of low amounts of H_2O_2 decreased insulin-stimulated glucose transport and GLUT4 translocation

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2-DG, 2-deoxy-D-[H^3]glucose; ANOVA, analysis of variance; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GSH, glutathione; LA, α-lipoic acid; LDH, lactate dehydrogenase; MAPK, mitogen-activated protein kinase; PBS, phosphate-buffered saline.

(19,20). Pretreatment with LA, at micromolar concentrations, protected the effects of insulin (21). Because the therapeutic effects of LA occur at plasma concentrations in the micromolar range (17,22), it is possible that protection against oxidative stress is one mechanism by which LA improves insulin action. However, the potential effect of LA on oxidative stress in muscle cells has not been evaluated.

In the present report, we investigated the effects of LA on cultured rat L6 muscle cells that had undergone oxidative stress. To improve both the responsiveness and sensitivity to insulin, we studied cells that were engineered to overexpress GLUT4 (23). In these cells, we find that oxidative stress inhibits insulin action on glucose transport. We now report that LA has little effect on nonstressed cells; however, in stressed cells, LA at micromolar concentrations restores responsiveness to insulin.

RESEARCH DESIGN AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM; 5.5 mmol/l D-glucose), fetal calf serum (FCS), penicillin, streptomycin, and amphotericin B were obtained from the University of California (San Francisco) Cell Culture Facilities. Glucose oxidase and vitamins C and E were purchased from Sigma (St. Louis, MO). 2-deoxy-D-[H^3]glucose (2-DG) was purchased from NEN Life Science (Boston, MA). The following reagents were gifts from the indicated organizations: insulin (Eli Lilly, Indianapolis, IN), LA (racemic LA, R-LA, and S-LA; Antibioticos, Rodano, Italy), and troglitazone (Parke-Davis Pharmaceuticals, Ann Arbor, MI).

Cell culture. L6 wild-type (L6 WT) cells and L6 GLUT4 cells (clone SG4–811) (23) were cultured (37°C, 5% CO_2) in DMEM, supplemented with 10% FCS, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. For glucose transport assays, cells were subcultured into 24-well cluster plates. L6 WT cells were plated at 10,000 cells/ml and allowed to differentiate spontaneously (7–10 days after plating), as described previously (24). L6 GLUT4 cells were plated at 40,000 cells/ml, and transport assays were performed at ~80% confluence. These cells did not require differentiation.

Glucose transport. L6 GLUT4 and L6 WT cells were incubated (in triplicate) in DMEM with 0.5% bovine serum albumin (BSA) for various times, and then placed in transport buffer consisting of 20 mmol/l HEPES (pH 7.4), 140 mmol/l NaCl, 5 mmol/l KCl, 2.5 mmol/l $MgCl_2$,1 mmol/l $CaCl_2$, and 0.1% (wt/vol) BSA. Next, insulin (1–1000 nmol/l) was added for 30 min, followed by 10 µmol/l 2-DG (1.0 µCi/ml) for 30 min at 37°C. Reactions were stopped by aspirating the media and thoroughly washing the monolayers with phosphate-buffered saline (PBS) containing 20 mmol/l D-glucose (at 4°C). Cells were solubilized in 0.03% (wt/vol) SDS, and radioactivity was determined by liquid scintillation counting. Data were expressed per milligram of lysate protein, which was determined using the bicinchoninic acid method (Pierce Chemical, Rockford, IL). Preliminary studies indicated that insulin stimulation of glucose transport in L6 WT cells was maximum when cells were serum starved for 4 h, whereas in L6 GLUT4 cells the maximum response occurred when cells were serum starved for 18 h.

Treatment with antioxidants. L6 GLUT4 cells were washed in DMEM supplemented with 0.5% BSA. Next, LA or other antioxidants were added, and cells were incubated for 18 h. Cells were then washed and incubated in 0.5 ml DMEM (phenol red-free) supplemented with 0.5% BSA, 100 mU/ml glucose oxidase, and 5 mmol/l D-glucose for 2 h. To measure the amount of H_20_2 generated, media were collected from triplicate wells, transferred to tubes containing 0.25 ml 50% (wt/vol) trichloroacetic acid, chilled on ice, and centrifuged (5000*g* for 10 min). Aliquots (1 ml) of the supernatant were added to 0.2 ml of 10 mmol/l ferrous ammonium sulfate and 0.1 ml of 2.5 mol/l potassium thiocyanate. Absorbance was measured spectrophotometrically at 491 nm using *t*-butyl hydroperoxide as a standard. Lactate dehydrogenase was measured in the culture medium using a colorimetric kit purchased from Sigma (Product No. 500C).

Reduced glutathione determination. Cells were treated to produce oxidative stress as described above. Next, cells were washed 3 times with PBS, scraped, and sonicated for 20 s. Then an equal volume of 10% (vol/vol) metaphosphoric acid was added to samples, incubated at room temperature for 5 min, and centrifuged for 5 min. Samples were assayed for glutathione (GSH) using a glutathione assay kit (Cayman Chemical, #703002).

p38 Mitogen-activated protein kinase. Cells were grown until 80% confluent, then treated to produce oxidative stress as described above. Next, cells were washed and solubilized, and 20 µg protein was loaded onto 8–16% Tris-glycine gel (Novex). Proteins were transferred to nitrocellulose, then incubated overnight with antiphospho p38 mitogen-activated protein kinase (MAPK; 1:1000). Signal was detected using a Phototope-HRP Western Detection Kit (New England Biolabs).

Statistical analyses. Data are expressed as means ± SE. Differences between means were assessed by Student's *t* test or one-way analysis of variance (ANOVA). Post hoc comparisons were performed using either a Dunnett's or Newman-Keuls test for multiple comparisons. Statistical significance was accepted at $P < 0.05$. All analyses (and graphics) were performed using GraphPad Prism (MS Windows version 3.02; GraphPad Software, San Diego, CA; www.graphpad.com).

RESULTS

Comparison of insulin effects on glucose transport in L6 WT and L6 GLUT4 cells. When GLUT4 was transfected and expressed in L6 cells, these cells were more responsive and more sensitive to insulin stimulation of glucose transport than L6 WT cells (Fig. 1*A* and *B*). There was a trend toward decreased basal 2-DG uptake in the L6 GLUT4 cells, although this difference was not statistically significant. Expression of GLUT4 in L6 cells did not affect the level of expression of GLUT1 (data not shown). In L6 WT cells, an effect of insulin was detected at 10 nmol/l, and maximal effects were detected at 1000 nmol/l. At these concentrations, insulin increased glucose transport by 50–70%. In contrast, in L6 GLUT4 cells, insulin stimulation was detected at 1.0 nmol/l, and maximal effects were detected at 100 nmol/l. In this concentration range, insulin typically increased glucose transport by 200–400%. The EC_{50} values for the L6 WT and L6 GLUT4 cells were 149 and 3 nmol/l, respectively (Fig. 1*B*). Accordingly, L6 GLUT4 cells were used in subsequent studies.

Direct effects of LA on glucose transport in L6 GLUT4 cells. We next investigated whether LA exerted a direct effect on glucose transport. L6 GLUT4 cells were exposed to LA for up to 18 h, followed by the addition of 100 nmol/l insulin for 30 min. Over the concentration range of 1–1,000 µmol/l, LA produced only a small effect on both basal and insulin-stimulated glucose transport (Fig. 2). In the absence of insulin, 1000 µmol/l LA stimulated 2-DG uptake by 65% ($P < 0.05$). In the presence of insulin (1 µmol/l), 1,000 µmol/l LA stimulated 2-DG uptake by ~22% (NS). At concentrations >1,000 µmol/l, cell death occurred.

Effects of LA on glucose transport in L6 GLUT4 cells exposed to oxidative stress. We next used glucose and glucose oxidase to generate H_2O_2, causing oxidative stress as previously described (21). Increasing the glucose oxidase concentration from 10 to 100 mU/ml caused a linear increase in H_2O_2 production (Fig. 3). Pretreatment of cells with 100 mU/ml of glucose oxidase produced an H_2O_2 concentration of 40–50 µmol/l. When cells were treated with this concentration of glucose oxidase, basal glucose transport was decreased slightly (but not significantly), whereas insulin-stimulated transport was nearly abolished ($P < 0.05$) (Fig. 4). Pretreatment with 300 µmol/l LA had a small, but not significant, stimulatory effect on basal glucose transport, but completely restored the responsiveness to insulin ($P < 0.05$).

To evaluate whether the protective effect of racemic LA on insulin-stimulated glucose transport could be attributed to the activity of a particular isomer, we studied the effects of the two individual isomers of LA, R and S, on oxidative stress–induced insulin resistance. For each isomer, a protective effect was observed at 30 µmol/l ($P < 0.05$), and a maximal effect was achieved at 1,000 µmol/l ($P < 0.001$) (Fig. 5). The

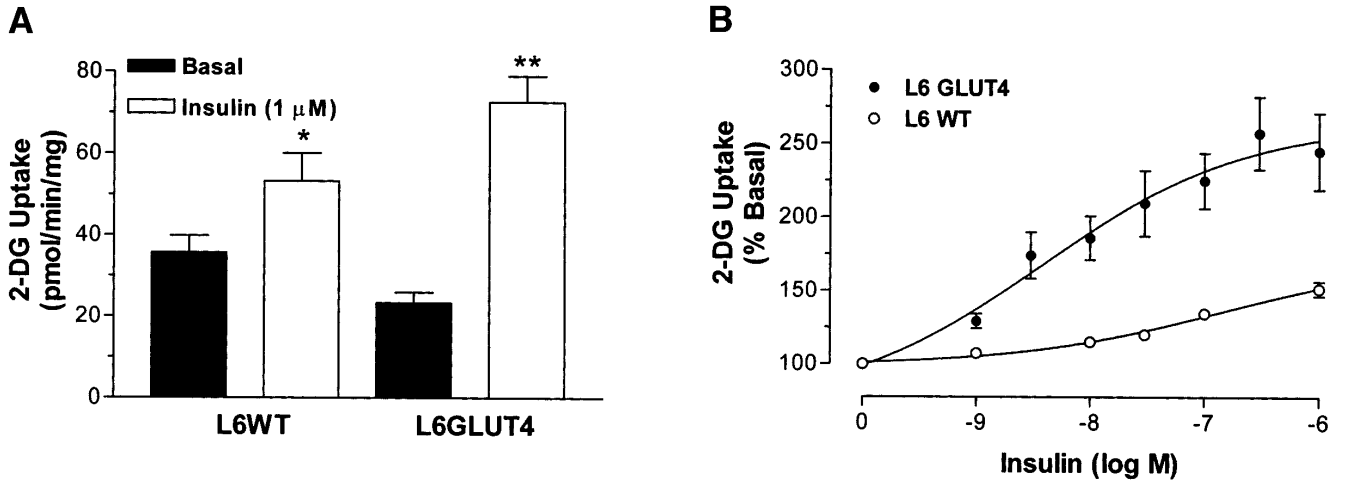

FIG. 1. Effects of insulin on 2-DG uptake in L6 WT and L6 GLUT4 cells. Cells were serum starved as described in RESEARCH DESIGN AND METHODS, washed, placed into transport buffer without glucose, and incubated in the absence or presence of insulin (1 μmol/l) for 30 min. Next, 2-DG was added for 30 min and uptake measured. *A*: Basal and maximal insulin-stimulated 2-DG uptake. Data represent means ± SE of three independent experiments. *P < 0.05 vs. L6 WT basal; **P < 0.001 vs. L6 GLUT4 basal (Student's t test, one-tailed, unpaired). There was a trend toward decreased basal 2-DG uptake in the L6 GLUT4 cells, although this difference was not statistically significant. *B*: Dosage-response of insulin. Basal 2-DG uptake for L6 WT and L6 GLUT4 was 35 ± 6 and 24 ± 4 pmol · min^{-1} · mg^{-1} protein, respectively. These values were set to 100% and others expressed as percent basal. The EC_{50} values for L6 WT and L6 GLUT4 cells were 149 and 3 nmol/l, respectively. Data represent means ± SE of four (L6 WT) or five (L6 GLUT4) independent experiments. For L6 WT and L6 GLUT4 cells, all insulin concentrations except 1 nmol/l were significantly different from the corresponding basal values (P < 0.05 for 3 nmol/l and P < 0.01 for all other concentrations [ANOVA and Dunnett's post hoc test]).

EC_{50} values for the R and S isomers were 96 and 131 μmol/l, respectively, but these values were not statistically different.

In addition to LA, exposure to another antioxidant, vitamin E, also afforded significant protection against oxidative stress–induced insulin resistance (Fig. 6). In cells treated with vitamin E (5 μmol/l), the response to insulin was nearly normalized. In contrast, vitamin C (300 μmol/l) and troglitazone (5 μmol/l), a thiazolidinedione possessing the vitamin E moiety, were ineffective at protecting against oxidative stress at the concentrations tested. There was a trend toward increased basal rates of 2-DG uptake in cells treated with LA and vitamins C and E, although this difference was not statistically significant.

Cell toxicity and intracellular GSH content. To determine whether H_2O_2 generation was causing cell toxicity or death, we measured the release of the cytosolic marker enzyme, lactate dehydrogenase (LDH). At 100 μmol/l H_2O_2, there was no significant increase in LDH activity released into the incubation medium (data not shown). The release of LDH activity was detected only at concentrations of H_2O_2 >1 mmol/l. Thus, under the experimental conditions of the present study, it is unlikely that the effects of LA could be attributed to simply protecting the cells from cell death.

Treatment of cells with 100 mU/ml of glucose oxidase and 5 mmol/l glucose for 2 h resulted in a reduction in the intracellular GSH content from 56.7 ± 10.7 to 32 ± 11.3 nmol/mg protein (P < 0.05) (Fig. 7), reflecting a condition of oxidative stress resulting from an alteration in intracellular redox state (21). To determine if LA could protect against the reduction in GSH content brought about by increased H_2O_2, cells were incubated for 18 h with racemic LA in the absence or presence of glucose oxidase. In cells pretreated with LA, GSH content was 68.3 ± 11.9 nmol/mg protein (NS vs. control) in cells not subsequently exposed to glucose oxidase and 52.0 ± 14.5 nmol/mg protein in cells that were treated with glucose oxidase. These results suggest that LA treatment provides protection against the oxidative stress–induced decrease in GSH content.

Protection against p38 MAPK activation. In L6 cells, it was reported recently that acute exposure to H_2O_2 activates p38 MAPK coincident with the inhibition of insulin action (25). The H_2O_2-induced effects could be effectively antagonized by two synthetic inhibitors of the p38 MAPK. To assess whether LA would also protect against H_2O_2-stimulated p38 MAPK activation, cells were incubated in the presence or absence of 300 μmol/l LA, followed by the addition of the H_2O_2-generating system. As reported by Blair et al. (25), H_2O_2 caused a

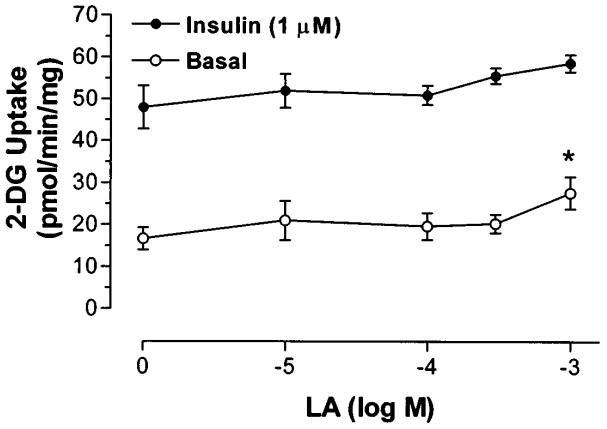

FIG. 2. Direct effects of LA on 2-DG uptake in L6 GLUT4 cells. L6 GLUT4 cells were preincubated for 18 h with racemic LA, and insulin-stimulated 2-DG uptake was measured as described in legend for FIG. 1. Data represent means ± SE of three independent experiments. *P < 0.05 vs. basal (Student's t test, one-tailed, unpaired). No significant difference for either data set was detected when data were analyzed by ANOVA.

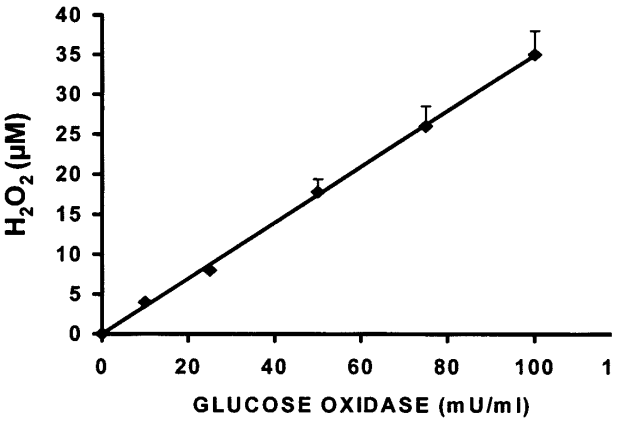

FIG. 3. The generation of H_2O_2 by glucose oxidase and glucose in L6 GLUT4 cells. L6 GLUT4 cells were serum starved as described in FIG. 1, washed, and placed into serum-free medium containing 5 mmol/l glucose and the indicated concentrations of glucose oxidase. The H_2O_2 concentration in the medium was measured after 60 min. Data represent means ± SE of three independent experiments (r = 0.998, P < 0.0001).

marked activation of p38 MAPK, as judged by the increase in p38 MAPK phosphorylation (Fig. 8; compare *lanes 1* and *2*). This effect was substantially blocked when cells were preincubated with LA (Fig. 8; compare *lanes 2* and *4*).

DISCUSSION

In the present study, we investigated the effects of LA on basal and insulin-stimulated glucose transport in cultured rat L6 myocytes. Prior studies in this cell type have demonstrated that insulin-stimulated glucose transport is mediated by the GLUT4 transporter (23,24,26), as is the case for skeletal muscle (27–30). In this study, we used L6 GLUT4 cells that had been engineered to overexpress GLUT4 (23). These L6 cells have two major advantages over L6 WT cells that express relatively low levels of GLUT4 (23,31): first, they are much more responsive and sensitive to insulin when compared with L6 WT cells; second, unlike L6 WT cells, L6 GLUT4 cells do not require differentiation from myoblasts into myotubes in order to express GLUT4. Thus there are significant benefits in terms of ease of cell culture and reproducibility of results.

In L6 GLUT4 cells, we found that LA had only small effects on cells that were not subjected to oxidative stress. When we exposed cells to oxidative stress using an H_2O_2-generating system (glucose and glucose oxidase), we found that insulin stimulation of glucose transport was nearly abolished. Pretreatment with LA for 18 h prevented this loss of insulin action. The beneficial effect of LA was detected at a concentration of 30 μmol/l, and a maximal effect was observed at 1,000 μmol/l. In 3T3-L1 adipocytes, similar results were reported with respect to the ability of LA to protect against the H_2O_2-induced loss of insulin-stimulated glucose uptake (21). Because therapeutic concentrations of LA fall within this micromolar range (17,18,22), it is possible that the protective effect of LA on insulin action in vitro is linked to its therapeutic effect in vivo.

One group has reported a direct effect of LA on glucose transport in cultured L6 rat muscle cells and mouse 3T3-L1 adipocytes (24). This effect was blocked by inhibitors of phosphatidylinositol 3-kinase, but was additive to the effect of insulin, suggesting that it was using some but not all of the

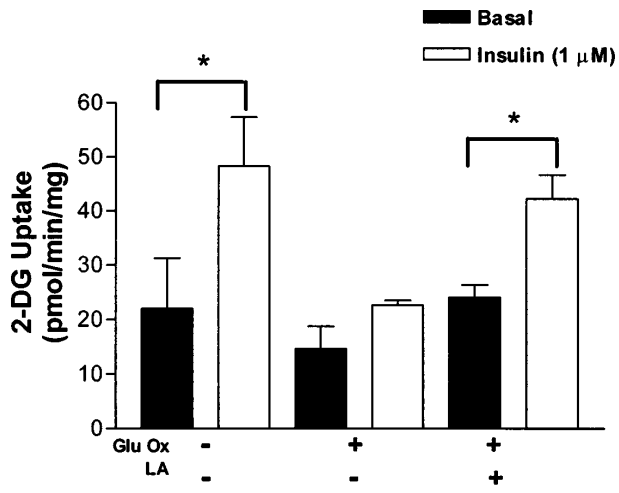

FIG. 4. Protective effect of LA on insulin-stimulated 2-DG uptake. L6 GLUT4 cells were preincubated for 18 h with 300 μmol/l racemic LA, washed, and then treated with glucose oxidase (100 mU/ml) and glucose (5 mmol/l) for 60 min. Cells were washed and incubated with 1 μmol/l insulin for 30 min, and 2-DG uptake was measured. Data represent means ± SE of three independent experiments. *P < 0.05 (ANOVA and Newman-Keuls post hoc test). In cells exposed to glucose oxidase and glucose (Glu Ox), there was no statistical difference between basal and insulin-stimulated 2-DG uptake. In the absence of LA, insulin-stimulated 2-DG uptake was significantly lower in cells exposed to glucose oxidase and glucose compared with insulin-stimulated 2-DG in untreated control cells (P < 0.05, ANOVA and Newman-Keuls post hoc test). In the presence of LA, insulin-stimulated 2-DG uptake was not significantly different in cells exposed to glucose oxidase and glucose compared with insulin-stimulated 2-DG in untreated control cells.

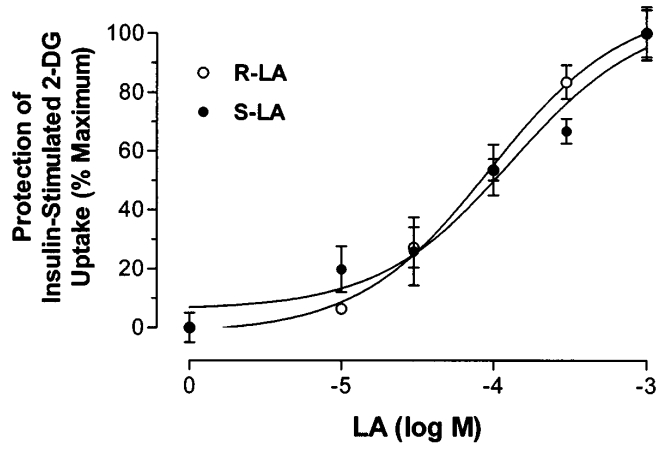

FIG. 5. Comparison of the protective effects of R and S isomers of LA on insulin-stimulated 2-DG uptake. L6 GLUT4 cells were preincubated for 18 h with increasing concentrations of either R-LA or S-LA, washed, and then treated with glucose oxidase (100 mU/ml) and glucose (5 mmol/l) for 60 min. They were then washed and incubated with 1 μmol/l insulin for 30 min, and 2-DG uptake was measured. Data represent means ± SE of three independent experiments. Basal 2-DG uptake was 28 ± 2.1 pmol · min^{-1} · mg^{-1} protein and was subtracted from the mean maximum R-LA and S-LA values (52.7 ± 6.1 and 52.0 ± 5.5 pmol · min^{-1} · mg^{-1} protein, respectively). The corrected values were set to 100%, and other values expressed as percent of the corresponding maximum. The EC_{50} values for R-LA and S-LA were 96 and 131 μmol/l, respectively (NS; Student's t test, two-tailed, unpaired). For R-LA and S-LA, all concentrations except 10 μmol/l were significantly different from the corresponding basal values (P < 0.05 for 30 μmol/l and P < 0.001 for all other concentrations; ANOVA and Dunnett's post hoc test).

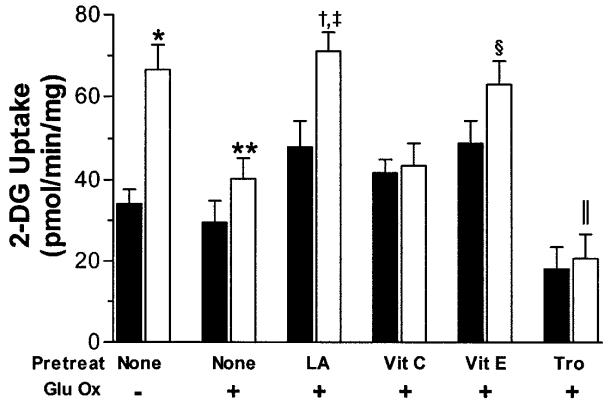

FIG. 6. Comparison of LA and other antioxidants on protecting insulin-stimulated 2-DG uptake. L6 GLUT4 cells were preincubated for 18 h with racemic LA (300 µmol/l), vitamin C (Vit C; 300 µmol/l), vitamin E (Vit E; 5 µmol/l), or troglitazone (Tro; 5 µmol/l); washed; and then treated with glucose oxidase (100 mU/ml) and glucose (5 mmol/l) for 60 min. Cells were then washed and incubated with 1 µmol/l insulin for 30 min, and 2-DG uptake was measured. Data represent means ± SE of six independent experiments. Data were analyzed by ANOVA and Newman-Keuls post hoc test. *P < 0.01 vs. control basal; **P < 0.05 vs. control insulin-stimulated; †P < 0.05 vs. Glu Ox + LA; ‡P < 0. 0.01 vs. Glu Ox + insulin; §P < 0.05 vs. Glu Ox + insulin; ‖P < 0.05 vs. Glu Ox + insulin. There was a trend toward increased basal rates of 2-DG uptake in the cells treated with LA and vitamins C and E, although this difference was not statistically significant.

insulin-signaling system. However, in that study, the LA effect on glucose transport was observed at 10-fold higher concentrations of LA (i.e., millimolar or greater) than the effective concentrations reported here and in a previous study (21). Similarly, a direct stimulatory effect on glucose transport in response to a millimolar concentration of LA was recently reported in isolated cardiac myocytes (32). In contrast, in both the present study with L6 cells and the earlier study with 3T3-L1 adipocytes (21), only a small direct effect of LA on glucose transport could be detected, whereas major effects were observed in cells that had been subjected to oxidative stress. Thus the relationship and significance of the direct effect exerted by millimolar concentrations of LA on cellular glucose transport to LA's therapeutic effects in patients remains to be defined. Our results are consistent with the observations of Jacob et al. (9) that administration of LA in vivo improved insulin-stimulated glucose transport in skeletal muscle only in the insulin-resistant obese Zucker (*fa/fa*) rats (which are under increased oxidative stress at the tissue level) (33) and not in the insulin-sensitive lean Zucker rats.

Synthetic LA exists as an ~50:50 mixture of two different isomers: the naturally occurring R isomer and the synthetic S isomer. In one study comparing the ability of the two isomers to directly stimulate glucose transport in vitro, R-LA was reported to be more potent than S-LA (24). In obese insulin-resistant Zucker rats, parenteral administration of R-LA improved both oxidative and nonoxidative glucose metabolism to a greater degree than did S-LA (11). However, in other studies, S-LA was reported to be equipotent or more potent than R-LA. For example, in studies of 3T3-L1 adipocytes, S-LA was more potent than R-LA in protecting against the inhibition of insulin action induced by oxidative stress (21). In the present study using muscle cells, we also compared the two isomers and found that both the R and S isomers were biologically active; each was able to protect against oxidative stress–induced insulin resistance. The potency of the two isomers was similar and not statistically different (96 vs. 131 µmol/l for R-LA EC_{50} and S-LA EC_{50}, respectively) (Fig. 5). Thus the biological activities of the individual enantiomers merit further study.

The mechanisms by which H_2O_2 and other mediators of oxidative stress cause insulin resistance are unknown. Similarly, the mechanisms by which LA offers protection against the H_2O_2-induced attenuation of insulin action are also unknown. A possible explanation for the inhibitory effect of H_2O_2 on insulin action is that it triggers an alteration of the cellular redox balance because of prolonged exposure to reactive oxygen molecules. The inhibitory effects of H_2O_2 have been reported to target the proximal steps in the insulin-signaling cascade, including the suppression of insulin-stimulated insulin receptor and insulin receptor substrate-1 tyrosine phosphorylation (20,34). Stress inducers, including H_2O_2, activate a variety of serine/threonine kinase cascades (35–37). Increased phosphorylation of insulin receptor substrates on discrete serine or threonine sites decreases the extent of their tyrosine phosphorylation and is consistent with impaired insulin action (38–44). Therefore, it is possible

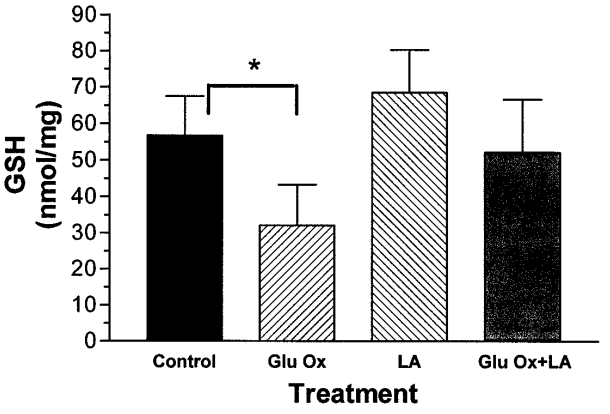

FIG. 7. Protective effect of LA on GSH levels. L6 GLUT4 cells were preincubated in the presence or absence of 300 µmol/l LA. Next, cells were washed, and 100 mU/ml glucose oxidase (Glu Ox) was added for 2 h. Cells were treated as described in RESEARCH DESIGN AND METHODS. Data represent means ± SE of three independent experiments. *P < 0.05 (ANOVA and Dunnett's post hoc test). There was a trend toward increased GSH in cells treated with LA compared with untreated control cells, although this difference was not statistically significant. In the presence of LA, GSH was not significantly different in cells exposed to glucose oxidase and glucose compared with GSH in untreated control cells.

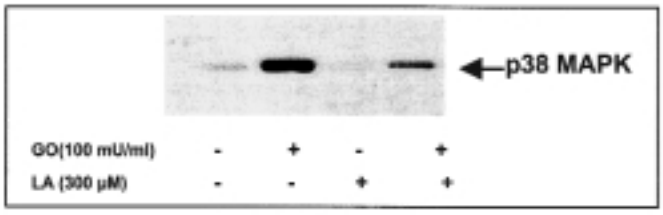

FIG. 8. Protective effect of LA on p38 MAPK activation. L6 GLUT4 cells were preincubated in the presence or absence of 300 µmol/l LA. Next, cells were washed, and 100 mU glucose oxidase (GO) was added for 2 h. Cells were solubilized and loaded on Tris-glycine gels; filters were probed with anti-phospho p38 MAPK antibody.

that one component of the inhibitory effect of H_2O_2 on insulin action might be mediated via the activation of inhibitory serine/threonine kinase activity.

Evidence to support this possibility has been provided in a recent study by Blair et al. (25). In L6 cells, it was reported that acute exposure to H_2O_2 activates p38 MAPK coincident with the inhibition of insulin action (25). The H_2O_2-induced effects were antagonized by two synthetic inhibitors of the p38 MAPK. Results from the present study in L6 GLUT4 cells confirmed that oxidative stress via H_2O_2 generation acutely stimulates p38 MAPK activity. This report, therefore, is the first to identify LA as an additional pharmacological agent capable of blocking the activation of this inhibitory kinase coincident with its ability to provide protection against oxidative stress–induced insulin resistance. It cannot be determined from this study if LA exerts its inhibitory effect on p38 MAPK directly via inhibition of kinase activity or indirectly via modulation of the cellular redox state (see below). Nonetheless, taken together, these results provide additional support for a link between the p38 MAPK–signaling pathway and the insulin-signaling pathway to regulate glucose transport in skeletal muscle cells.

In muscle cells undergoing oxidative stress, the specific sites in the insulin-signaling pathway that are protected by LA are unknown. However, under equivalent experimental conditions, Tirosh et al. (20) reported that the protection afforded by LA against oxidative stress–induced insulin resistance in 3T3-L1 adipocytes involved the preservation of insulin-induced cellular redistribution of IRS-1 and PI 3-kinase. It is likely, therefore, that a similar mechanism occurs in L6 cells.

A potential explanation for the protective effects of LA on H_2O_2-induced insulin resistance may be related to its ability to preserve the intracellular redox balance, acting either directly or through other endogenous antioxidants, such as glutathione. The limiting factor in glutathione synthesis is the bioavailability of intracellular cysteine. LA and other antioxidants generate intracellular cysteine from extracellular cystine and thus maintain reduced glutathione levels (45). Rudich et al. (21) and other researchers (45) have reported that in 3T3-L1, human erythrocytes, human Jurkat cells, and others, LA pretreatment maintains the cellular reduced glutathione concentration in response to subsequent oxidative stress and have suggested that preservation of the normal reduced glutathione concentration is the major mode of action of LA. We found that pretreatment with LA protects against the fall in reduced glutathione caused by oxidative stress. Thus this protective effect of LA occurs in multiple cell types.

When we studied other antioxidants and related compounds, vitamin E also protected cells against oxidative stress, whereas vitamin C and troglitazone were ineffective. Whether vitamin E and LA possess similar modes of action is unknown. It is of interest that troglitazone, a clinically effective insulin sensitizer that contains the vitamin E moiety, was ineffective in protecting cells (46).

One possible explanation for the effects of oxidative stress on insulin signaling is via a lowering of ATP levels. For several reasons, we believe that changes in this parameter did not occur under our experimental conditions. First, several other groups studying H_2O_2-mediated oxidative stress have not observed significant falls in intracellular ATP levels (47,48). Second, if a decrease in ATP levels did occur, basal glucose transport would have increased because of the activation of the 5′ AMP-kinase (49). This enzyme is activated by increases in the AMP:ATP and creatine:phosphocreatine ratios (50). Third, the study of Blair et al. (25) demonstrated that under conditions of oxidative stress, inhibition of p38 MAPK restored the insulin effect on glucose transport. This result would have been extremely unlikely if ATP was substantially decreased.

In summary, we found that in L6 muscle cells, micromolar concentrations of LA protect the insulin-signaling system from oxidative stress. These findings are in agreement with similar studies using 3T3-L1 adipocytes. Thus, in two of the major insulin-sensitive target tissues, LA action in vitro possesses potent protective effects that are in concert with its reported therapeutic effects in vivo. Taken together, these studies support the concept that the antioxidant actions of LA are an important feature of its clinical efficacy.

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