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Lipoic acid Inhibits JNK Pathway and IRS-1 Serine Phosphorylation while Improving Insulin Sensitivity in Rat L6 Muscle Cells Exposed to Oxidative Stress.

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ABSTRACT

The antioxidant α-lipoic acid (LA) has been shown to improve insulin sensitivity in insulin resistant subjects. However, the molecular mechanism of action of LA is unknown. In the present study, we investigated the effect of LA on insulin action, redox balance and redox sensitive serine kinase JNK pathway in cultured rat L6 muscle cells exposed to oxidative stress. L6 myotubes were incubated with LA (300µM) for 18 hrs and then treated with H_2O_2 generating (glucose/glucose oxidase) system for 12 hrs. Treatment of L6 muscle cells with H_2O_2 decreased the insulin stimulated insulin receptor subatrate-1 (IRS-1) tyrosine phosphorylation and glucose transport. Treatment with H_2O_2 also impaired the intracellular redox balance, activated the redox sensitive JNK pathway and increased the IRS-1 serine phosphorylation. Pretreatment with LA preserved the redox balance, inhibited the JNK pathway and improved the insulin action in L6 muscle cells exposed to oxidative stress. The present study shows for the first time that LA inhibit JNK pathway and IRS-1 serine phosphorylation while improving insulin sensitivity in oxidative stress induced insulin resistance. Further studies, which explore the effect of LA on redox sensitive serine kinase pathways and insulin signaling in insulin resistant animal models and humans will add our understanding of the molecular basis of insulin resistance and will open novel therapeutic areas for the treatment of insulin resistance and its resultant pathogenesis.

Keywords: Oxidative stress, Insulin resistance, Lipoic acid, JNK pathway Insulin receptor substrate-1(IRS-1)

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INTRODUCTION

Insulin is an anabolic hormone with powerful metabolic effects. The events after insulin binding to its receptor are highly regulated and specific. Insulin receptor is a transmembrane tyrosine kinase that is expressed as a tetramer in a $\alpha_2\beta_2$ configuration. Insulin binding to α subunit eventuate autophosphorylation of specific tyrosine residues of β subunits. Autophosphorylation results in activation of the tyrosine kinase activity of insulin receptor. The activated insulin receptor kinase phosphorylates the tyrosine residues of various endogenous substrates, including insulin receptor substrate - 1 (IRS-1). Tyrosine phosphorylation of IRS-1 activates two major pathways - the mitogen activated protein kinase ((MAPK) cascade, mostly associated with the transcriptional and mitogenic effects of insulin, while the phosphatidyl inositol 3 kinase (PI3K) pathway is engaged with the hormone's metabolic effects [1]. One of the primary actions of insulin is maintenance of glucose homeostasis. Insulin regulates glucose homeostasis mainly by reducing hepatic glucose output and increasing the rate of glucose uptake by striated muscle and fat cells. Insulin enhances the glucose entry into target cells by promoting the translocation of intracellular vesicles containing the glucose transporter isoform 4 (GLUT4) to plasma membrane via PI3K pathway [2].

Insulin resistance occurs when normal circulating concentrations of the hormone fails to regulate body glucose homeostasis. Resistance to insulin stimulated glucose uptake is a common derangement in type 2 diabetes. Experimental evidences have attributed a defect in post receptor signal transduction as the major cause of insulin resistance [3]. There is considerable evidence that hyperglycemia results in the generation of reactive oxygen species (ROS). Oxidative stress resulting from increased production of ROS plays a key role in the pathogenesis of late diabetic complications [4]. Numerous studies have linked the role of oxidative stress in the pathogenesis of insulin resistance [5, 6]. When rat L6 muscle cells and mouse 3T3L1 adipocytes were exposed to oxidative stress, insulin stimulated glucose uptake was inhibited [7, 8]. Many studies have shown that the ROS induces activation of multiple stress sensitive serine kinase cascades and their role in the pathogenesis of insulin resistance [9,10]. One such major intracellular serine kinase target for oxidative stress is C-Jun-N-terminal kinase (JNK). JNK pathway is the member of serine/threonine protein kinase superfamily of mitogen activated protein kinase (MAPK) pathway. The JNK pathway is activated by variety of exogenous and endogenous stress signals including oxidative stress, osmotic stress, proinflammatory cytokines, heat shock and UV irradiation [11]. The activation of JNK pathway is known to interfere with insulin action [12]. It has been reported that JNK pathway is activated in various tissues under diabetic condition [13-15]. Thus, it is likely that the oxidative stress induced activation of JNK pathway is a crucial mediator in the progression of insulin resistance.

Through in vitro and animal models of insulin resistance, it has been found that antioxidants, especially α-lipoic acid (LA) improve insulin sensitivity [16,17]. In patients with type 2 diabetes, LA improves insulin stimulated glucose disposal [18]. In cultured 3T3L1 adipocytes and L6 muscle cells, LA at micromolar concentrations protects against the oxidative stress induced insulin resistance [7]. Because the therapeutic effect of LA is at micromolar range [19], it is possible that protection against oxidative stress is one of the mechanisms by

which LA improves insulin action. However, the potential mechanism by which LA protects against the oxidative stress induced insulin resistance is not known. It is worth investigating the above mentioned events because these studies could yield new insights into the molecular basis of insulin resistance and may help in identifying new pharmaceutical targets for the treatment of type 2 diabetes. In view of the above, the present study was designed to investigate the effect of LA on insulin action and JNK pathway on rat L6 muscle cells exposed to oxidative stress.

MATERIALS AND METHODS

Materials:

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, glucose oxidase, α-lipoic acid, 1,1-diphenyl -2-picryl hydrazyl (DPPH), insulin, sodium vandate, phenyl methyl sulfonyl fluoride, aprotinin, leupeptin, okadaic acid, and all other chemicals were purchased from Sigma Chemicals (St.Louis, MO). 2 deoxy – ^{14} C – D glucose was purchased from Amersham Life Sciences. IRS –1, JNK_1, P-JNK_1 and phospho tyrosine antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Phospho serine antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Protein-A agarose slurry was purchased from Bangalore Genei (Bangalore, India).

Cell culture:

Rat L6 myoblasts (American Type Culture Collection) were cultured (37 ^{0} C, 5% CO_2) in growth medium (DMEM, 10 % FCS, 100 units/ml penicillin, 100 μg/ml streptomycin). L6 myoblasts were allowed to differentiate into myotubes in differentiation medium (DMEM, 2% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin) as described previously [20].

Hydrogen peroxide treatment:

Rat L6 myotubes were incubated with LA (300 μM) in DMEM supplemented with 0.5% BSA for 18 hrs. After LA treatment, myotubes were washed with DMEM supplemented with 0.5% BSA and incubated in DMEM (phenol red free) supplemented with 0.5 % BSA, 25mu/ml glucose oxidase and 5 mmol/l glucose for 12 hrs. H_2O_2 generated by this system was estimated from the media collected at different time intervals using Amplex red – hydrogen peroxide/peroxidase assay kit (Molecular Probes, USA). Creatinine Kinase (CK) activity was measured in the culture medium using colorimetric kit (Teco Diagnostics, USA) in 550 Express plus autoanalyser (Ciba Corning Diagnostics, USA). H_2O_2 induced cytotoxicity was measured using 3-(4,5-Dimethylthiazol -2-yl)-2,5-diphenyltetrazolium - bromide (MTT) as described previously [21].

Determination of glucose transport into myotubes:

Rat L6 myotubes were treated with LA(18 hrs) followed by H_2O_2 (12 hrs) as described above. After glucose / glucose oxidase treatment, L6 myotubes were washed with DMEM

supplemented with 0.5% BSA and then incubated with serum free DMEM for 3 hrs. After serum starvation, insulin (100nmol/l) was added to the serum free medium and further incubated for 30 min. Myotubes were washed with HBS (140 nmol/l NaCl, 20 mmol/l Hepes pH 7.4, 5.0 mmol/l KCl, 2.5 nmol/l $MgSO_4$, 1.0 mmol/l $CaCl_2$) and glucose uptake was measured as described previously [22]. Briefly, after insulin stimulation, myotubes were incubated with 2 Deoxy – ^{14} C – D glucose (0.5 μCi/ml) in HBS for 10 min. Radioactive medium was aspirated rapidly and cells were washed in ice-cold isotonic saline (0.9% NaCl). Cells were lysed in 0.05M NaOH and radioactivity was determined by liquid scintillation counting. Proteins in lysates were estimated by the method of Bradford [23]. Non-specific glucose uptake was determined in the presence of cytochalasin B (50μmol/l) an inhibitor of facilitative glucose transport and was subtracted from total uptake.

Antioxidant assays:

Rat L6 myotubes were treated with LA (18 hrs) followed by H_2O_2 (12 hrs) as described above and lysed in 0.5 ml of PBS by repeated freezing and thawing. Total antioxidant status of cell lysates was quantified by trolax equivalent antioxidant capacity assay (TEAC) using 1,1-Diphenyl –2-Picryl Hydrazyl (DPPH) as described previously [20]. Briefly, total antioxidant activities of lysates were determined spectrophotometrically at 517 nm by quantifying the decrease in the absorbance of free radical DPPH after the addition of cell lysates. The antioxidant capacities of samples were measured against a trolax standard and expressed as TAEC. Reduced glutathione concentration in cell lysates was estimated using Ellman's reagent (5-5,Dithio bis –2- nitrobenzoic acid) as described by Beutler et al [23].

Insulin signaling analysis:

Rat L6 myotubes were treated with LA (18 hrs) followed by H_2O_2 (12 hrs) as described above and incubated with serum free DMEM for 30 minutes. After serum starvation, insulin (100 nmol/l) was added to the serum free medium and incubated for 15 minutes. Myotubes were washed with ice-cold PBS and scraped into ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1mM sodium vandate, 1mM phenyl methyl sulfonyl fluoride, 1 mM aprotinin, 1mM leupeptin, 0.5 μg/ml okadaic acid). Cell lysates were centrifuged at 12,000g for 15 minutes at 4^0C. Protein content of the supernatant collected was estimated by the method of Bradford [24]. Insulin signaling analysis was performed by immunoprecipitation and immunoblotting as decribed previously [25]. Briefly, cell lysates (500 μg of protein) were incubated overnight at 4^0C with insulin receptor and IRS-1 antibodies. The immune complexes were captured by adding 50μl of protein A-agarose beads for 2 hours at 4^0C. Immune complexes were pelleted at 12,000g for 15 minutes at 4^0C and washed three times with cell lysis buffer. The immune complexes were suspended in Laemmli sample buffer [26] and boiled for 5 minutes. Protein A agarose was removed from the denatured proteins by centrifugation at 12,000g for 15 minutes at 4^0C. The supernatant was resolved by 8.0 % SDS-PAGE, electrotransferred onto nitrocellulose membrane. Proteins were immunoblotted with antibody specific to phosphorylated tyrosine and immunoblot was stripped of bound antibodies and then reprobed with antibody specific to phosphorylated

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serine. Protein bands were visualized by enhanced chemiluminescence method using Amersham ECL – kit (Amersham Life Sciences, Buckingham, UK). Bands were scanned using a densitometer (Bio-Rad, Model GS-710, USA) and quantified by Quantity 1 software (Bio-Rad, USA). The band densities of tyrosine and serine phosphorylation of insulin receptor and IRS-1 were normalized with corresponding amount of immunoprecipitated proteins.

Analysis of JNK pathway:

Rat L6 myotubes were treated with LA (18 hrs) followed by H_2O_2 (12 hrs) as described above. Cell lysates (100 μg of protein) were resolved by 12% SDS-PAGE, electrotransferred onto nitrocellulose membrane and immunoblotted with antibody specific to phospho JNK_1. Further, the membrane was stripped of bound antibodies and then reprobed with antibody specific to JNK_1. Band detection and quantification was done as described above.

Statistical analysis:

Data are expressed as mean ± SEM. Differences between means were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-test. A 'P' value less than 0.05 was considered as statistically significant.

RESULTS

Lipoic acid protects insulin stimulated glucose uptake in rat L6 muscle cells exposed to oxidative stress.

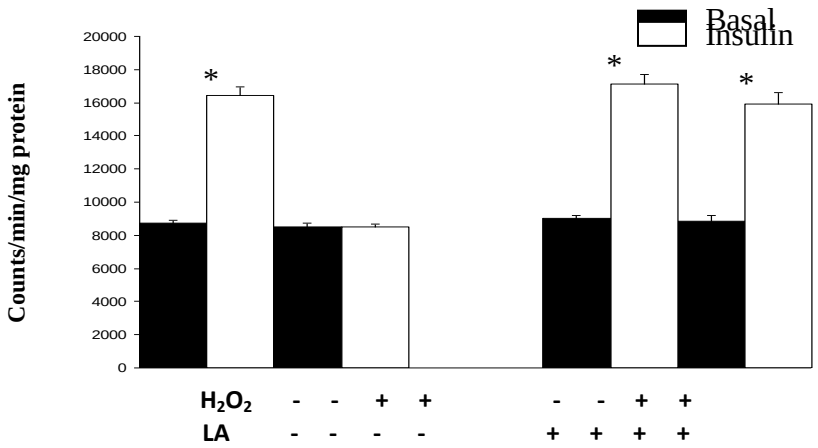

Fig.1. Lipoic acid protects insulin stimulated glucose uptake in rat L6 cells exposed to oxidative stress: Rat L6 myotubes were pretreated with LA acid for 18 hrs and then exposed to H_2O_2 for 12 hrs. 2 – deoxy-^{14}C - glucose uptake was measured in myotubes after insulin stimulation as described in the methods. Data represent mean ± SEM of three independent experiments done in triplicate. *P<0.001 in comparison to basal glucose uptake.

Addition of 25mu/ml glucose oxidase, 5mmol/L glucose for 12 hrs resulted in a H_2O_2 concentration in the medium that achieved a study state of 40 ± 10 μmol/L after 30 minutes.

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When cells were incubated at this concentration of glucose oxidase, basal uptake was not affected, whereas insulin stimulated glucose uptake was decreased significantly (Fig.1).Pretreatment with LA had no effect on basal glucose transport, but restored insulin stimulated glucose uptake in cells exposed to oxidative stress.

Lipoic acid inhibits IRS-1 serine phosphorylation and improves insulin stimulated IRS-1 tyrosine phosphorylation in rat L6 muscle cells exposed to oxidative stress.

H_2O_2 treatment significantly decreased insulin stimulated IRS-1 tyrosine phosphorylation [Fig.2 (a)] compared to cells not exposed to H_2O_2. In addition, H_2O_2 treatment significantly increased the basal serine phosphorylation of IRS-1 [Fig.2(b)] compared to cells not exposed to H_2O_2. Pretreatment with LA inhibited the oxidative stress induced IRS-1 serine phosphorylation and improved the insulin stimulated IRS-1 tyrosine phosphorylation in L6 muscle cells treated with H_2O_2.

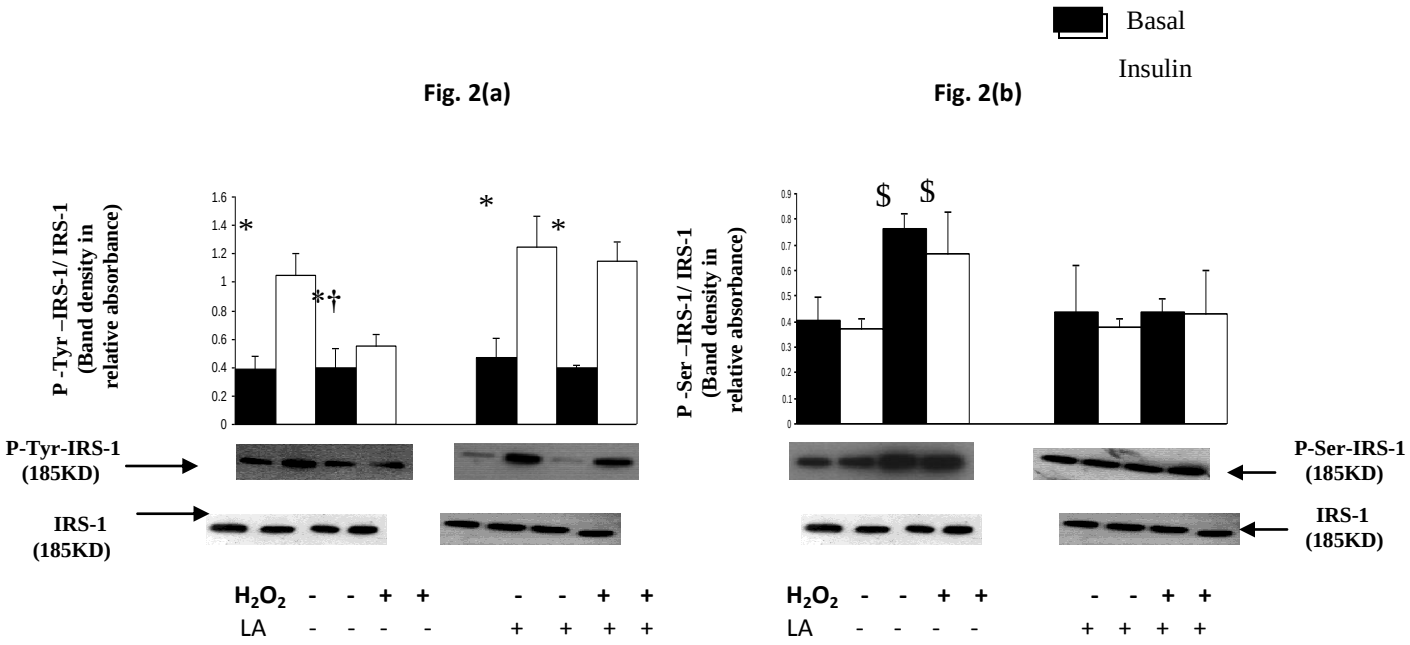

Fig.2. Lipoic acid preserves insulin signaling in rat L6 cells exposed to oxidative stress: Rat L6 myotubes were pretreated with LA for 18 hrs and then exposed to H_2O_2 for 12 hrs. Insulin signaling analysis was done in cell lysates after insulin stimulation.

Fig. 2 (a); Effect of oxidative stress and LA on IRS-1 tyrosine phosphorylation.

Fig.2 (b); Effect of oxidative stress and LA on IRS-1 serine phosphorylation. A representative immunoblot of three independent experiments is shown. Results shown are mean ± SE of three experiments. *P < 0.001 compared to basal. †P< 0.001 compared to insulin stimulated H_2O_2 untreated cells. $^{\$}$P<0.001 compared to H_2O_2 untreated cells.

Lipoic acid restores redox balance and inhibits redox sensitive JNK pathway in rat L6 muscle cells exposed to oxidative stress.

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H_2O_2 treatment to L6 cells significantly decreased the total antioxidant capacity [Fig. 3(a)], reduced glutathione levels [Fig.3(b)] and activated the redox sensitive serine kinase JNK pathway (Fig.4). Pretreatment with LA restores the redox balance and inhibits the oxidative stress induced activation of JNK pathway. To determine whether H_2O_2 treatment cause cell toxicity, we performed the cytotoxicity (MTT) assay and measured the release of skeletal muscle cytosolic marker enzyme creatinine kinase (CK) in culture medium after incubation with glucose oxidase. The MTT assay and CK activity in the culture medium did not show any significant difference between the groups (data not shown).Thus, under the experimental conditions of the present study, the effect of LA is due to the protection against the oxidative stress, not due to simply protecting the cells from cell death.

Fig. 3(a). **Fig. 3(b).**

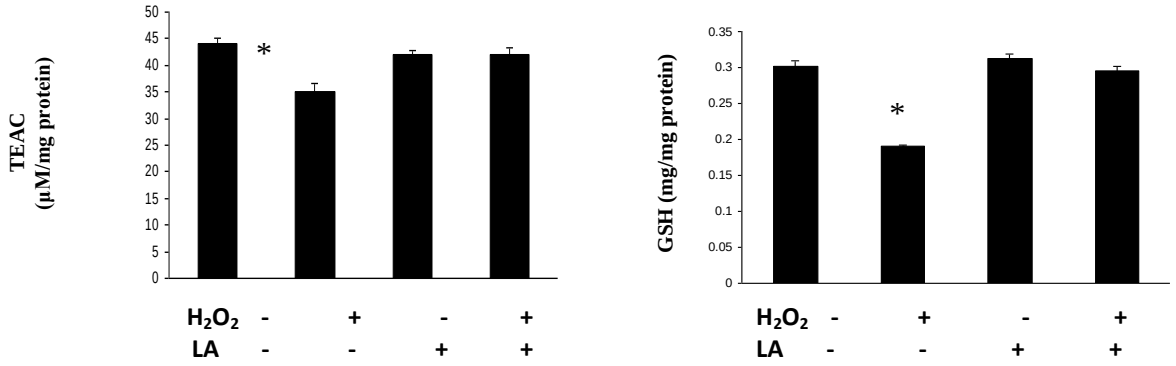

Fig.3. Protective effect of lipoic acid on redox balance in rat L6 muscle cells exposed to oxidative stress: Rat L6 myotubes were pretreated with LA for 18 hrs and then exposed to H_2O_2 for 12 hrs. Total antioxidant capacity [Fig.3(a)] and reduced glutathione [Fig.3(b)] were estimated in the cell lysates as described n the methods. Data represent mean ± SEM of three independent experiments done in triplicate. *P < 0.001 compared to other groups.

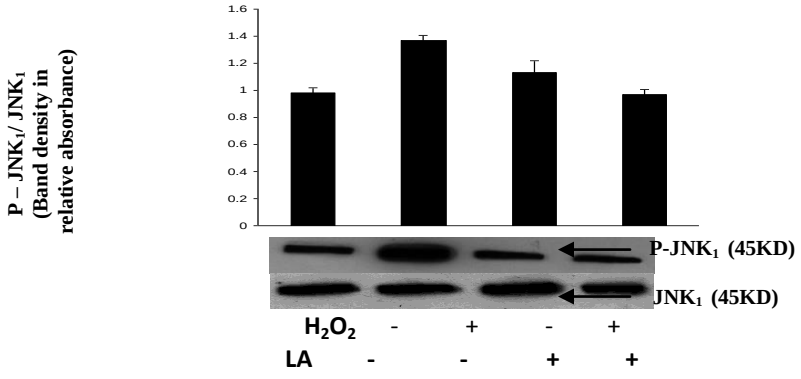

Fig.4. Lipoic acid prevents activation of JNK pathway in rat L6 cells exposed to oxidative stress: Rat L6 myotubes were pretreated with LA for 18 hrs and then exposed to H_2O_2 for 12 hrs. Western blotting analysis was performed to analyze JNK pathway. A representative immunoblot of three independent experiments is shown. Results shown are mean ± SE of three experiments. *P < 0.001 compared to other groups.

DISCUSSION

In the present study, we investigated the effect of LA on insulin stimulated glucose uptake, proximal insulin signaling and JNK pathway in cultured rat L6 muscle cells exposed to oxidative stress. When L6 cells were exposed to oxidative stress using a H_2O_2 generating system, the insulin stimulated glucose transport and IRS-1 tyrosine phosphorylation were decreased. Oxidative stress also increased the IRS-1 serine phosphorylation, impaired intracellular redox balance and activated the redox sensitive serine kinase – JNK pathway. LA pretreatment restored the intracellular redox balance, inhibited JNK pathway and improved insulin action in L6 muscle cells exposed to oxidative stress. Even though, previous reports [7,8,27] are in support of our findings that oxidative stress inhibits insulin stimulated glucose uptake and insulin signaling, evidence have also been provided that H_2O_2 has an insulinomimetic effect [28,29]. These discrepancies can be partly explained by the differences in the experimental design and in concentration of reagent used. The insulin like action of H_2O_2 is reported in cells which were exposed to H_2O_2 for a shorter duration (< 60 min) at millimolar concentrations [30]. In the present study we exposed the rat L6 muscle cells for a chronic period (12hrs) under low grade oxidative stress (40 $\pm$ 10 µM H_2O_2). Under the experimental conditions oxidative stress and LA treatment did not have significant effect on basal glucose transport. Whereas oxidative stress decreased the insulin stimulated glucose transport and LA pretreatment restored it. Estrada et al [31] reported a direct effect of LA on glucose transport in cultured rat L6 muscle cells and mouse 3T3L1 adipocytes. However in that study, the effect of LA on glucose transport was observed at 10-fold higher concentrations of LA (i.e., millimolar or greater) used in the present study and previous studies [7, 32]. Similarly, a direct stimulatory effect on glucose transport in response to millimolar concentrations of LA was reported in isolated cardiac myocytes [33]. In contrast, in the present study with L6 cells and previous studies [7,32], there were no/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. In the insulin resistant obese Zucker (*fa/fa*) rats (which are under increased oxidative stress at tissue level) LA administration improved insulin stimulated glucose transport in skeletal muscle, but no effect was noticed in insulin sensitive lean Zucker rats [34]. Thus the insulin sensitizing property of LA is associated with the protection against the oxidative stress.

The mechanisms by which oxidative stress cause insulin resistance and how LA offers protection against oxidative stress induced insulin resistance is not known. 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. The inhibitory effect of H_2O_2 has been reported to target the proximal steps in the insulin signaling cascade, including the suppression of insulin stimulated IRS-1 tyrosine phosphorylation [27,35]. In the present study, oxidative stress increases the basal serine phosphorylation of IRS-1 and decreased the insulin stimulated IRS-1 tyrosine phosphotylation. Recent studies have documented increased IRS-1 serine phosphorylation as a potential molecular mechanism for insulin resistance [36-38]. In patients with type 2 diabetes and animal models of insulin resistance, IRS-1 serine phosphorylation was found to be increased [39]. Numerous agents that induce insulin resistance, such as TNF-α, okadaic acid, platelet-derived growth factor and angiotensin II all increases IRS-1 phospho serine content. IRS-1 contains more than 30 serine residues, which provide a potential site for phosphorylation by number of serine kinases linked to insulin

resistance [40]. Serine phosphorylation of IRS-1 impairs its interaction with the juxtamembrane domain of insulin receptor and thus renders IRS-1 as a poorer substrate for insulin receptor kinase and decrease the insulin stimulated tyrosine phosphorylation [41].

ROS can function as signaling molecules and activates number of redox sensitive serine/theronine kinase cascades linked to insulin resistance [6]. Identification of such redox sensitive serine kinases involved in the pathogenesis of insulin resistance is an intense area of research. Our results show that oxidative stress in rat L6 muscle cells impairs redox balance and activates the redox sensitive serine kinase - JNK pathway. Evidence from cellular models and transgenic animals demonstrated the role of JNK in the pathogenesis of insulin resistance. Support for the importance of JNK pathway in insulin resistance is provided by the results of gene knockout experiments in mice. Suppression of JNK pathway improves insulin sensitivity in db/db mice and sucrose fed rats [42]. Several studies show inhibitors of JNK pathway improves insulin signaling and insulin sensitivity [43]. In this regard, we propose that treatment of L6 cells with H_2O_2 impairs the intracellular redox imbalance and activates redox sensitive JNK cascade. Oxidative stress induced activation of JNK in turn phosphorylates IRS-1 and increases phospho serine content of IRS-1. Increased IRS-1 serine phosphorylation inhibits its interaction with insulin receptor and decreases insulin stimulated IRS-1 tyrosine phosphorylation. This results in insulin resistance in cells exposed to oxidative stress. The protective effect of LA on oxidative stress induced insulin resistance could be related to its ability to preserve the intracellular redox balance and thereby preventing the activation of redox sensitive JNK pathway and IRS-1 serine phosphorylation. In conclusion, more detailed understanding of the role of oxidative stress and antioxidants on redox sensitive Ser/Thr kinase cascades in humans and animal models of insulin resistance might open novel therapeutic targets for the treatment of type 2 diabetes and its complications.

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