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α-Lipoic acid decreases thiol reactivity of the insulin receptor and protein tyrosine phosphatase 1B in 3T3-L1 adipocytes

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Abstract

α-Lipoic acid is known to increase insulin sensitivity *in vivo* and to stimulate glucose uptake into adipose and muscle cells *in vitro*. In this study, α-lipoic acid was demonstrated to stimulate the autophosphorylation of insulin receptor and glucose uptake into 3T3-L1 adipocytes by reducing the thiol reactivity of intracellular proteins. To elucidate mechanism of this effect, role of protein thiol groups and H_2O_2 in insulin receptor autophosphorylation and glucose uptake was investigated in 3T3-L1 adipocytes following stimulation with α-lipoic acid. α-Lipoic acid or insulin treatment of adipocytes increased intracellular level of oxidants, decreased thiol reactivity of the insulin receptor β-subunit, increased tyrosine phosphorylation of the insulin receptor, and enhanced glucose uptake. α-Lipoic acid or insulin-stimulated glucose uptake was inhibited (i) by alkylation of intracellular, but not extracellular, thiol groups downstream of insulin receptor activation, and (ii) by diphenylene iodonium at the level of the insulin receptor autophosphorylation. α-Lipoic acid also inhibited protein tyrosine phosphatase activity and decreased thiol reactivity of protein tyrosine phosphatase 1B. These findings indicate that oxidants produced by α-lipoic acid or insulin are involved in activation of insulin receptor and in inactivation of protein tyrosine phosphatases, which eventually result in elevated glucose uptake into 3T3-L1 adipocytes.

Keywords: α-Lipoic acid; Insulin; 3T3-L1 adipocytes; Glucose uptake; Insulin receptor; Protein tyrosine phosphatase; Thiols

1. Introduction

α-Lipoic acid, a disulfide derivative of octanoic acid, functions as the prosthetic group for several redox reactions catalyzed by cellular α-keto-acid dehydrogenases, such as the pyruvate dehydrogenase complex [1]. It is known that α-lipoic acid has the ability to alter the redox status of cells, and interact with thiols and other antioxidants [2]. In clinical trials, α-lipoic acid improved glucose metabolism in diabetic patients [3], and was found beneficial in the treatment of diabetic neuropathy [4]. α-Lipoic acid treatment also increased insulin sensitivity in type 2 diabetic patients [3,5,6] and enhanced glucose transport into skeletal muscle isolated from both obese and lean Zucker rats [7]. In a comprehensive series of studies, Klip and co-workers have demonstrated that α-lipoic acid increases tyrosine phosphorylation and/or the activity of several components of the insulin signaling pathway including the IR, insulin receptor substrate-1, type I PI3-K, protein kinase B/Akt1, and p38 mitogen-activated protein kinase [8,9]. Based on these findings, it has been proposed that α-lipoic acid stimulates glucose uptake into fat cells by activating the insulin signaling pathway, which enhances the translocation and intrinsic activity of glucose transporter-4 (Glut4) [9]. Although activation of IR may explain the increased activity of its downstream signaling components, the underlying mechanism of IR activation by α-lipoic acid has not been elucidated.

Accumulating evidence suggests that oxidant signals might integrally be involved in the regulation of the insulin signaling pathway. Treatment of IR-transfected Chinese hamster ovary cells with antioxidants such as NAC or butylated hydroxyanisole inhibited insulin responsiveness, whereas partial inhibition of glutathione metabolism, which intracellularly induces a mild oxidative stress condition,

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Abbreviations: AV, acetovanillone; *p*CMPS, *p*-chloromercuriphenylsulfonate; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DHLA, α-dihydrolipoic acid; DPI, diphenylene iodonium; GSH, glutathione; IR, insulin receptor; LA, α-lipoic acid; MBB, maleimidobutyrylbiocytin; NAC, *N*-acetyl-L-cysteine; NEM, *N*-ethylmaleimide; PAO, phenylarsine oxide; PI3-K, phosphoinositol-3-kinase; PTP, protein tyrosine phosphatase.

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stimulated IR tyrosine phosphorylation when measured *in vitro* [10]. A similar increase in IR kinase activity was observed following cell treatment with hydrogen peroxide (H_2O_2) [11,12]. Moreover, oxidation of critical cysteine residues in the IR β-subunit results in the increase of its intrinsic tyrosine kinase activity, whereas low concentration of dithiothreitol (DTT) inactivates the IR kinase, supporting the importance of oxidation of critical thiol groups in activation of the insulin signaling pathway [13]. Agents such as H_2O_2, Cu^{2+} and diamide that readily oxidize sulfhydryls to disulfides, markedly enhanced 3-*O*-methylglucose uptake in brown fat cells [14]. IR has also been demonstrated to couple, via $G\alpha_{i2}$, to the NADPH-dependent H_2O_2 generating system, which upon insulin stimulation produces H_2O_2 in 3T3-L1 adipocytes [15]. Insulin-dependent H_2O_2 production is associated with a decreased PTP activity [16] and is also found essential for the activation of PI3-K [17]. These findings suggest that redox signals are involved in the regulation of both the early tyrosine phosphorylation cascade and downstream insulin signaling events.

Recently, we have reported that the oxidized isoforms of α-lipoic acid stimulate glucose uptake into 3T3-L1 adipocytes by changing the intracellular redox status toward an oxidizing condition [18]. It is hypothesized that α-lipoic acid may alter the thiol reactivity of redox sensitive component(s) of the insulin signaling pathway based on its oxidant property. Here, the action of α-lipoic acid on the thiol groups of the IR and PTP1B in 3T3-L1 adipocytes was investigated.

2. Materials and methods

2.1. Chemicals

Insulin, 3-isobutyl-1-methylxanthine, dexamethasone, DPI, AV, and protease inhibitor cocktail solution were purchased from Sigma–Aldrich Chemical Co. 2-Deoxy-[^{3}H]-D-glucose was obtained from NEN Life Science Products. Protein A/G agarose and MBB were from Calbiochem. Monoclonal (29B4) or polyclonal (C-19) anti-IR β-subunit and anti-PTP1B antibodies were from Santa Cruz Biotech Inc. 4G10 anti-phosphotyrosine antibody was from Upstate Biotechnology. ECL detection system was from Amersham Pharmacia Biotech, Inc. Cell culture reagents and other chemicals were from Life Technologies. Racemic α-lipoic acid (99% purity) was provided by BASF AG.

2.2. Preparation of α-lipoic acid and pharmacological agents

Twenty-millimolar (20 mM) stock solution of racemic α-lipoic acid was freshly prepared in 20 mM HEPES, pH 7.4. Two micromolar (2 μM) of insulin stock was prepared in 10% (v/v) acetic acid, and the pH was adjusted to 7.4 with 1 N NaOH. Stock solutions of DCFH-DA, wortmannin, PAO and DPI were prepared in DMSO. Stock solution of AV was prepared in ethanol. All other agents were dissolved in distilled water. When the pharmacological agents were used, DMSO or ethanol was also added to the control cells as vehicle at the same concentrations. The final concentration of DMSO or ethanol in the cell culture medium did not exceed 0.1% (v/v).

2.3. Cell culture and 2-deoxy-[^{3}H]-D-glucose uptake

Mouse 3T3-L1 fibroblasts were grown to confluence and were differentiated as previously described [14]. Confluent 3T3-L1 cells or fully differentiated adipocytes (8–10 days after differentiation when more than 90% of cells exhibited the adipocyte phenotype) were used in the glucose uptake assay. Insulin was added just before start of the glucose uptake assay. The rate of glucose uptake was measured for 10 min at 37° using 2-deoxy-[^{3}H]-D-glucose as described earlier [18].

2.4. Measurement of intracellular reactive oxygen species

Intracellular oxidants were detected using oxidant-sensitive fluorescent probe $DCFH_2$-DA. Cells were equilibrated in HBSS for 2 hr, preincubated with DPI (20–200 μM) for 30 min, and then stimulated with α-lipoic acid for the indicated times. DCFH-DA (20 μM) was added 15 min before the cell harvest. Cells were scraped and the intracellular fluorescence intensity was determined using FACScalibur after exclusion of dead cells by propidium iodide (25 μg/mL) staining.

2.5. Intracellular glutathione

Cells seeded in 12-well plates were incubated with 250 μM of α-lipoic acid 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° until analysis by HPLC. Immediately before the assay, samples were thawed, vortexed and then centrifuged at 15,000 *g* for 2 min at 4°. 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, USA) as described previously [19].

2.6. Cell lysis and immunoprecipitation of insulin receptor and protein tyrosine phosphatase 1B

3T3-L1 adipocytes in T_{75} flasks or 100 mm dishes were serum-starved for 4 hr, washed with ice-cold PBS containing 1 mM of Na_3VO_4 and lysed in IR lysis buffer (pH 7.2) consisting of 50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P 40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 25 mM NaF, 1 mM sodium orthovanadate, and

0.25% protease inhibitor cocktail solution (Sigma). After centrifugation, the clear supernatants were incubated with 2 μg/mL of anti-IR β or anti-PTP1B antibody overnight or 4 hr at 4°, respectively. The immune complexes were collected by incubating the lysates with protein A/G overnight at 4°, then washed with IR lysis buffer twice, and separated as described below.

2.7. Western blotting

Cytosolic proteins or the immune complexes were separated by 10% SDS–PAGE and electro-transfered to a PVDF membrane. The membrane was blocked by either 1% BSA or 5% skim milk for 1 hr, and immunoblotted using rabbit or mouse polyclonal IgG against IR β-subunit, PTP1B, or anti-phosphotyrosine antibody (4G10). Immunoreactive bands were visualized using the Amersham ECL detection system. Protein was measured using a bicinchoninic acid assay solution.

2.8. Analysis of free thiol groups of the insulin receptor and protein tyrosine phosphatase 1B

The level of thiol-biotinylated IR β-subunit or PTP1B was measured as previously described [20]. 3T3-L1 adipocytes in T_{75} culture flasks were treated with insulin (100 nM) or α-lipoic acid (250–500 μM) for 30 min, washed twice with ice-cold PBS and immediately maintained in incubation buffer containing 20 mM HEPES, pH 7.5, 125 mM NaCl, 5 mM KCl, and 11.1 mM glucose for 5 min at 4°. MBB (100 μM) was added and the cells were incubated for another 10 min at 4°. L-Cysteine (4 mM) was subsequently added to remove excess MBB. The cell supernatant was removed and the flasks were rapidly immersed in liquid nitrogen. Cells were lysed in the IR lysis buffer, and the lysates were immunoprecipitated with either anti-IR β antibody or PTP1B antibody as described above.

The immune complexes were washed three times with IR lysis buffer and twice with washing buffer containing 0.1% Triton X-100 and 50 mM HEPES, pH 7.4. The immune complexes were separated by 10% SDS–PAGE, and electro-transfered to a PVDF membrane (Millipore Corp.). The membrane was blocked with either 1% BSA or 5% skim milk for 1 hr and immunoblotted by streptavidin coupled to horseradish peroxidase (HRP-ST, Vector Lab. Inc.) for 1 hr at RT. The biotinylated protein sulfhydryl groups were detected using the Amersham ECL detection system.

2.9. Protein tyrosine phosphatase activity assay

Assay for total PTP activity was performed as previously described [21] with minor modifications. Cells in 6-well plates were serum-starved in HBSS (200 mM NaCl, 5 mM KCl, 700 μM Na_2HPO_4, 1.2 mM $NaHCO_3$, 27.5 mM Tris, 1.87 mM $CaCl_2$, 800 μM $MgSO_4$ and 12 mM glucose, pH 7.4) for 2 hr, and stimulated with α-lipoic acid, H_2O_2, insulin, or NAC. Cell lysates were prepared in 300 μL of TES buffer (20 mM Tris, 1 mM EDTA, 8.73% sucrose, 150 mM NaCl, 1 mM DTT, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor, 10 μg/mL bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, pH 7.4).

PTP activity in cell lysates was determined by measuring the hydrolysis of *p*-nitrophenyl phosphate (*p*NPP). Cell lysate (80 μg) in 50 μL of TES buffer was added to 450 μL of reaction mixture consisting of 37.5 mM sodium acetate (pH 5), 150 mM NaCl, 2.5 mM EDTA, 0.1% bovine serum albumin, 2 mM DTT and 50 mM *p*NPP, and incubated for 30 min at 30°. The reaction was stopped by adding 500 μL of 2 M KOH, and the absorbance was measured at 405 nm. Nonspecific absorbance was corrected by subtracting the absorbance at 405 nm determined in the absence of cell lysate.

2.10. Data presentation

Data were reported as mean ± SD of at least three independent experiments performed in triplicate. Differences between means were assessed by ANOVA. The minimum level of significance was set at $P < 0.05$.

3. Results

3.1. Stimulation of glucose uptake by α-lipoic acid and insulin

In 3T3-L1 adipocytes, the rate of basal glucose uptake was 5.2 ± 2.1 nmol/min/mg protein. Adding insulin (100 nM) with ^{3}H-glucose without preincubation could strongly increase the glucose uptake into the cells by 4.7 ± 1.4-fold. Pretreatment of 3T3-L1 adipocytes with α-lipoic acid (500 μM) for 1 hr increased glucose uptake relative to the basal by 6.7 ± 0.9-fold (Fig. 1). No additive effect was found when cells were stimulated with α-lipoic acid and insulin in combination.

The rate of basal glucose uptake in undifferentiated confluent monolayers of 3T3-L1 cells was 2.0 ± 0.5 nmol/min/mg protein. Insulin stimulation non-significantly increased glucose uptake by 1.3 ± 0.3-fold over the basal level, whereas α-lipoic acid treatment (500 μM) for 1 hr led to significant increase (1.9 ± 0.2-fold) of glucose uptake into 3T3-L1 pre-adipocytes. Wortmannin, a PI3-K inhibitor, completely inhibited α-lipoic acid- or insulin-stimulated glucose uptake into either adipocytes or undifferentiated 3T3-L1 cells (Fig. 1).

3.2. Effect of α-lipoic acid and insulin on intracellular redox status and thiol reactivity of IR β-subunit

No change in cellular GSH levels was observed over 2 hr post-incubation of cells with 250 μM of α-lipoic acid

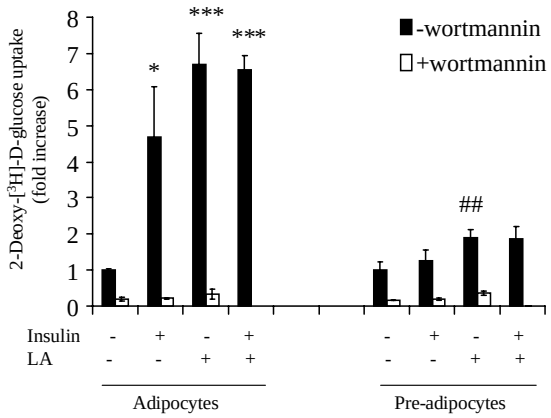

Fig. 1. Effect of insulin or α-lipoic acid on glucose uptake into differentiated and undifferentiated 3T3-L1 cells. 3T3-L1 adipocytes or undifferentiated confluent 3T3-L1 cells were treated with 500 μM of α-lipoic acid (LA) for 1 hr or stimulated with 100 nM insulin just before start of the glucose uptake assay. LA + insulin indicates that the cells were treated with a combination of α-lipoic acid and insulin as specified above. Wortmannin (200 nM) was added to the cells 1 hr before addition of insulin or α-lipoic acid. The extent of glucose uptake into the cells was expressed as fold increase compared to the basal rate; *, *** or ## significantly different ($P < 0.05$, $P < 0.001$ or $P < 0.01$) from the basal glucose uptake into differentiated (*, ***) or undifferentiated (##) 3T3-L1 cells, respectively.

(Fig. 2A). In addition, depletion of intracellular GSH synthesis using buthionine sulfoximine (BSO, 250 μM), a γ-glutamylcysteine synthase inhibitor, did not inhibit and slightly increased α-lipoic acid-stimulated glucose uptake

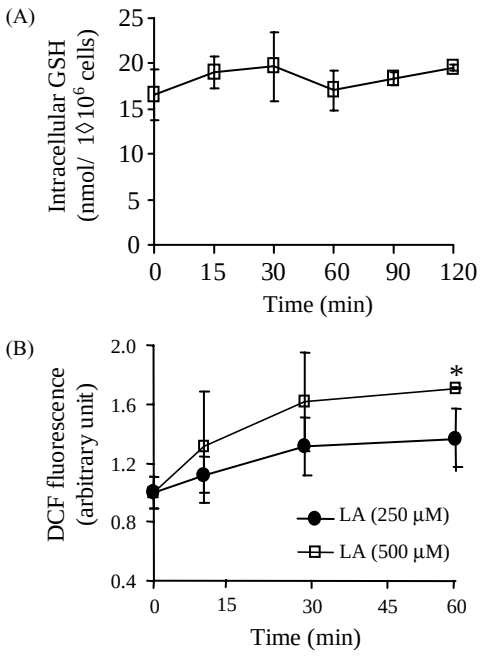

Fig. 2. Effect of α-lipoic acid on intracellular redox status of 3T3-L1 adipocytes. (A) 3T3-L1 adipocytes were incubated with 250 μM of α-lipoic acid (LA) for indicated times and intracellular GSH was measured by HPLC as described in Section 2. (B) Cells were incubated with 250 or 500 μM of α-lipoic acid for up to 60 min. DCF fluorescence was determined by flow cytometry as described in Section 2; * significantly different ($P < 0.05$) from the basal.

(data not shown). However, treatment of cells with 250 or 500 μM of α-lipoic acid significantly increased $DCFH_2$ oxidation within 60 min as estimated by the elevated DCF fluorescence (Fig. 2B).

α-Lipoic acid or insulin treatment of 3T3-L1 adipocytes increased tyrosine phosphorylation of IR β-subunit both in total cell lysate (Fig. 3A) and in IR-immunoprecipitated complex (Fig. 3B), while basal phosphorylation was not detectable. H_2O_2, a strong oxidant, could also induce noticeable amount of IR tyrosine phosphorylation (Fig. 3B). The observed increase in the tyrosine phosphorylation of IR β-subunit upon stimulation with α-lipoic acid, insulin or H_2O_2 was accompanied by a decrease in the thiol reactivity of IR β-subunit (Fig. 4A) as well as total cell lysate (Fig. 4B), while DTT, a thiol-reducing agent, did not decrease the free thiol level.

3.3. Modulation of α-lipoic acid-stimulated glucose uptake by thiol-reactive agents

Various well-known thiol-reactive agents were employed to explore the role of free thiol groups in α-lipoic acid- or insulin-stimulated IR autophosphorylation and glucose uptake. NEM and *p*CMPS are thiol alkylating agents, which covalently bind to low-molecular weight and protein thiols, whereas PAO reacts specifically with vicinal sulfhydryl groups to form stable dithioarsine rings [22].

Pretreatment of 3T3-L1 adipocytes with cell-permeable thiol-reactive agents, NEM or PAO, completely abolished α-lipoic acid- or insulin-stimulated glucose uptake (Table 1). On the other hand, pretreatment of the cells with *p*CMPS, a cell-impermeant thiol-alkylating agent non-significantly decreased insulin-stimulated glucose uptake and significantly potentiated α-lipoic acid-stimulated glucose uptake into 3T3-L1 adipocytes. Neither of these thiol-reactive agents, however, prevented insulin or α-lipoic acid-stimulated autophosphorylation of IR β-subunit (Fig. 5), though they increased basal phosphotyrosine levels of cellular proteins.

Table 1
Effect of alkylation/ligation of intra- or extracellular thiol groups on α-lipoic acid or insulin-stimulated glucose uptake

	Glucose uptake (%)	
	Insulin	LA
No treatment	100 ± 15.5	100 ± 3.5
N-Ethylmaleimide	−1.71 ± 0.01	−0.16 ± 0.43
Phenylarsine oxide	−1.12 ± 6.36	2.72 ± 6.25
p-Chloromercuriphenylsulfonate	72.34 ± 11.59	198.4 ± 45.82*

3T3-L1 adipocytes were pretreated with NEM (1 mM for 5 min), PAO (20 μM for 1 hr), or *p*CMPS (50 μM for 1 hr). Cells were stimulated with either α-lipoic acid (LA, 250 μM for 30 min) or 100 nM insulin added just before start of the glucose transport assay. Glucose uptake was expressed as percent of glucose uptake rate in cells stimulated with LA or insulin in the absence of the thiol-modifying agents.

* Significantly different from LA-stimulated glucose uptake in the absence of *p*CMPS ($P < 0.05$).

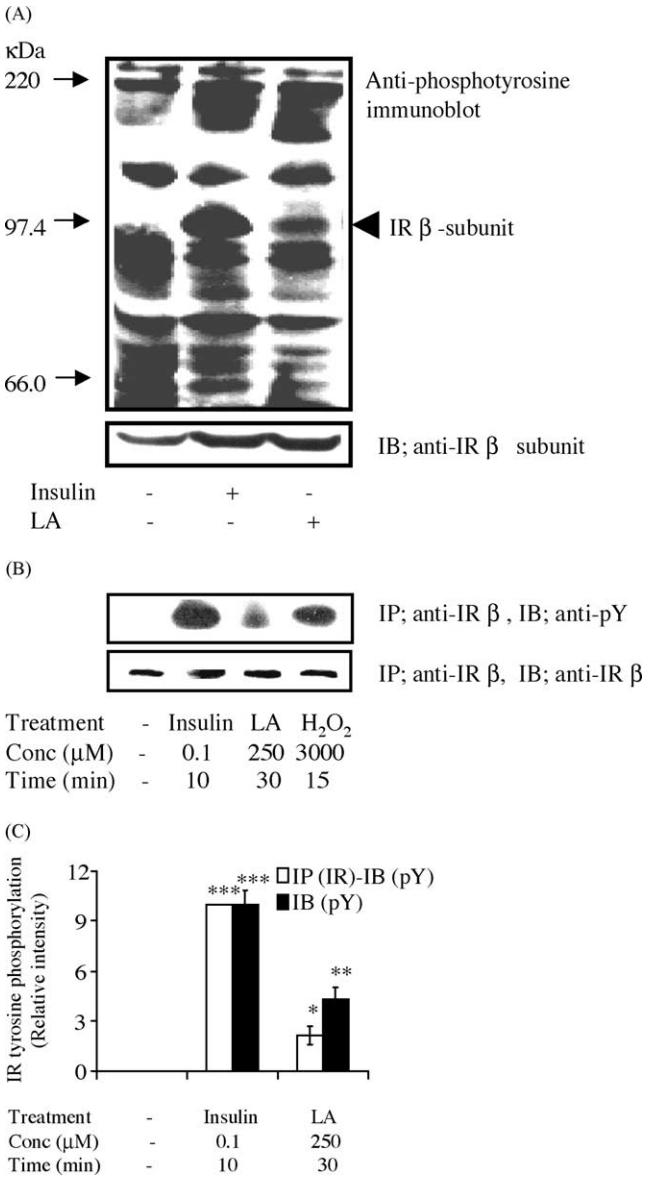

Fig. 3. Effect of insulin or α-lipoic acid on tyrosine phosphorylation of total cell lysates (A) or of immunoprecipitated insulin receptor β-subunits (B). (A) 3T3-L1 adipocytes were treated with insulin (100 nM) for 10 min or α-lipoic acid (LA, 500 μM) for 30 min, lysated and the same amount of proteins were separated and immunoblotted as described in Section 2 using anti-phosphotyrosine or anti-IR β-subunit antibody. (B) Cells were treated with insulin, α-lipoic acid (LA) or H_2O_2, lysated and immunoprecipitated with anti-IR β-subunit antibody as described in Section 2. The immunoprecipitated IR β-subunits were separated, and immunoblotted using indicated antibodies. (C) Each bar represents mean ± SD of three independent experiments. Band intensities were expressed relative to the intensity of the band in insulin-treated group, which was set at 10; *, ** or *** significantly different ($P < 0.05$, $P < 0.01$ or $P < 0.001$) from control. IR, anti-IR β-subunit antibody, pY, anti-phosphotyrosine antibody.

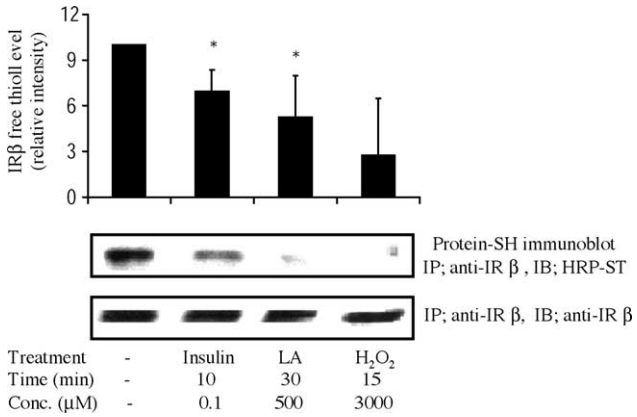

Fig. 4. Effect of insulin or α-lipoic acid on thiol reactivity of the insulin receptor β-subunit. Cells were stimulated with insulin, α-lipoic acid (LA) or H_2O_2, treated with 100 μM of MBB for 10 min and lysed. The immunoprecipitated IR β-subunits were loaded onto 10% SDS–PAGE gel, and free, reduced thiols were detected as described in Section 2. Each bar represents mean ± SD of three independent experiments. Band intensities were expressed relative to the intensity of control band, which was set at 10; * significantly different ($P < 0.05$) from control.

3.4. Role of NADPH oxidase in α-lipoic acid- or insulin-stimulated glucose uptake

Two NADPH oxidase inhibitors DPI [23] and AV [24] were used to evaluate the role of cellular H_2O_2 production in α-lipoic acid- or insulin-stimulated glucose uptake and IR autophosphorylation. DPI is reported to inhibit phagocyte NADPH oxidase by capturing an electron from the reduced heme iron of flavocytochrome *b* to become a free radical, which irreversibly phenylates the flavin or the adjacent amino acids [25]. AV, a methoxy-substituted catechol inhibits the assembly of phagocyte NADPH oxidase by interfering with the intracellular translocation of its cytosolic components [24].

Pretreatment of cells with DPI non-specifically increased basal glucose uptake (Fig. 6A). AV, however, did not affect the rate of basal glucose uptake (Fig. 6B). Pretreatment of 3T3-L1 adipocytes with DPI or AV inhibited α-lipoic acid- or insulin-stimulated glucose uptake in a dose-dependent manner. DPI, but not AV, also inhibited α-lipoic acid- or insulin-stimulated tyrosine phosphorylation of IR β-subunit (Fig. 7). However, DPI did not inhibit α-lipoic acid-stimulated increase of $DCFH_2$ oxidation, whereas it slightly attenuated insulin-stimulated oxidant production (Table 2).

Table 2
Effect of NADPH oxidase inhibitor, DPI, on α-lipoic acid- or insulin-stimulated intracellular oxidant production

	DCF fluorescence (arbitrary unit)	
	−DPI	+DPI
Basal	1.00 ± 0.01	1.02 ± 0.05
Insulin	1.83 ± 0.02***	1.60 ± 0.05#
LA	1.36 ± 0.01***	1.16 ± 0.15

Cells were pretreated with 20 or 200 μM of DPI for 1 hr and then stimulated with 100 nM of insulin for 15 min or 500 μM of α-lipoic acid (LA) for 30 min, respectively. Flow cytometric measurement of DCF fluorescence was performed as described in Section 2.

*** Significantly different from basal ($P < 0.001$).

Significantly different from insulin-treated group ($P < 0.05$).

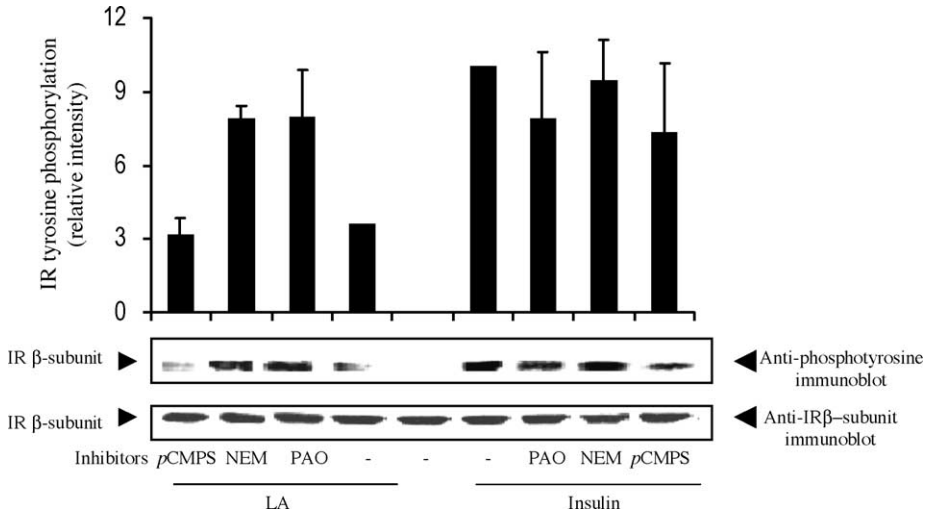

Fig. 5. Effect of alkylation/ligation of intra- or extracellular thiol groups on insulin or α-lipoic acid-induced IR tyrosine phosphorylation. 3T3-L1 adipocytes were pretreated with NEM (100 μM for 30 min), PAO (20 μM for 1 hr), or *p*CMPS (50 μM for 1 hr). Cells were then stimulated with either insulin (100 nM for 10 min) or α-lipoic acid (LA, 500 μM for 30 min). Anti-phosphotyrosine antibody was used to determine the level of protein tyrosine phosphorylation in total cell lysates. Each bar represents mean ± SD of three independent experiments. Band intensities were expressed relative to intensity of the band in insulin-treated group, which was set at 10. Presence of same amount of IR β-subunit in each lane was confirmed by using anti-IR β antibody.

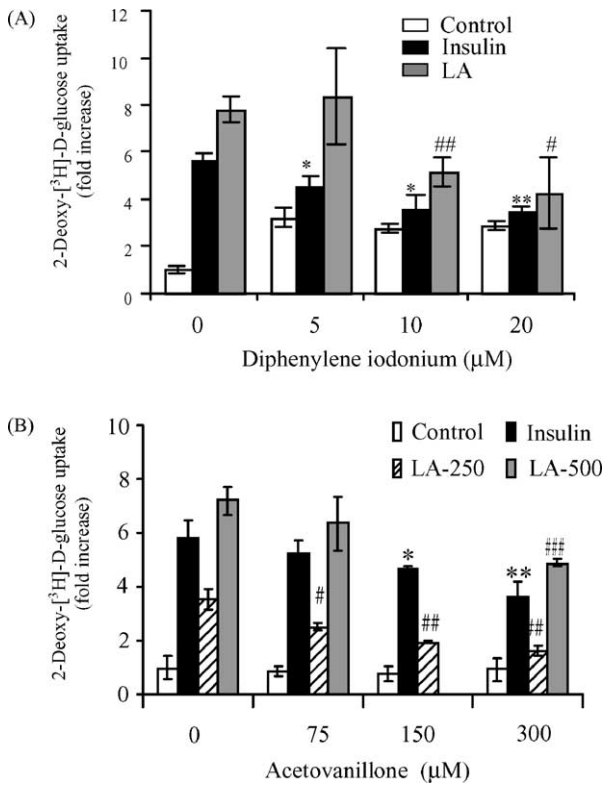

Fig. 6. Effect of NADPH oxidase inhibitors on insulin- or α-lipoic acid-stimulated glucose uptake. 3T3-L1 adipocytes were pretreated, at indicated concentrations, with (A) DPI for 30 min or (B) AV for 1 hr. The cells were subsequently stimulated with insulin (100 nM, 10 min) or with α-lipoic acid (500 μM, 1 hr for (A) and LA-500 in (B) or 250 μM, 30 min for LA-250 in (B)). Glucose uptake was expressed as fold increase compared to basal; * or ** is significant at $P < 0.05$ or $P < 0.01$ compared to insulin-stimulated cells in the absence of the inhibitors, and #, ## or ### is significant at $P < 0.05$, $P < 0.01$ or $P < 0.001$ compared to α-lipoic acid-stimulated cells in the absence of the inhibitors.

3.5. Inhibition of total PTP activity and alteration of PTP1B thiol reactivity by α-lipoic acid

Pretreatment of 3T3-L1 adipocytes with α-lipoic acid, insulin, or H_2O_2 significantly inhibited total cellular PTP activity, whereas NAC, a well-known antioxidant, increased the activity (Fig. 8A). The observed inhibition of the cellular PTP activity upon treatment with α-lipoic acid or H_2O_2 was also associated with decreased thiol reactivity of PTP1B, a key negative regulator of the tyrosine phosphorylation cascade integral to the insulin signaling pathway (Fig. 8B).

4. Discussion

This study demonstrates that α-lipoic acid increases intracellular oxidant levels, modifies free thiol groups of IR and PTP1B, stimulates IR tyrosine phosphorylation and inhibits total PTP activity. The fact that thiol reactivity of IR as well as PTP1B decreased upon α-lipoic acid or insulin stimulation strongly supports the role of oxidants in activation of IR, which eventually results in elevated glucose uptake into 3T3-L1 adipocytes.

4.1. Effect of insulin and α-lipoic acid on glucose uptake, cell redox status, and activation of the insulin receptor

As previously reported [8,9,18], α-lipoic acid increased uptake of glucose into 3T3-L1 adipocytes. Although α-lipoic acid (2.5 mM) was reported to increase insulin-stimulated glucose uptake in L6 myotubes [26], no additive

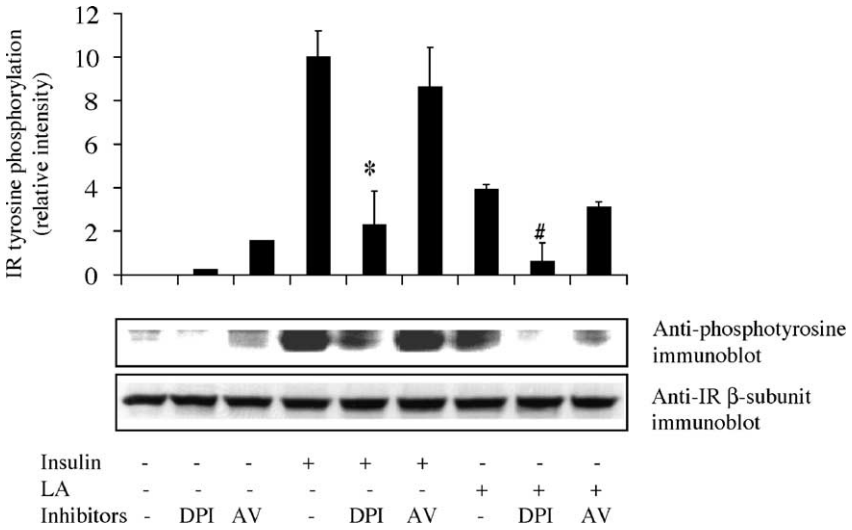

Fig. 7. Effect of the NADPH oxidase inhibitors on insulin- or α-lipoic acid-stimulated insulin receptor phosphorylation. 3T3-L1 adipocytes were pretreated with DPI (20 μM for 30 min) or AV (300 μM for 1 hr), and stimulated with either insulin (100 nM for 10 min) or α-lipoic acid (LA, 500 μM for 30 min). The anti-phosphotyrosine antibody was used to determine the phosphotyrosine level of the IR β-subunit. Each bar represents mean ± SD of three independent experiments. Band intensities were expressed relative to the intensity of the band in insulin-treated group, which was set at 10; * or # is significant at $P < 0.05$ compared to insulin- or α-lipoic acid-stimulated cells in the absence of the inhibitors.

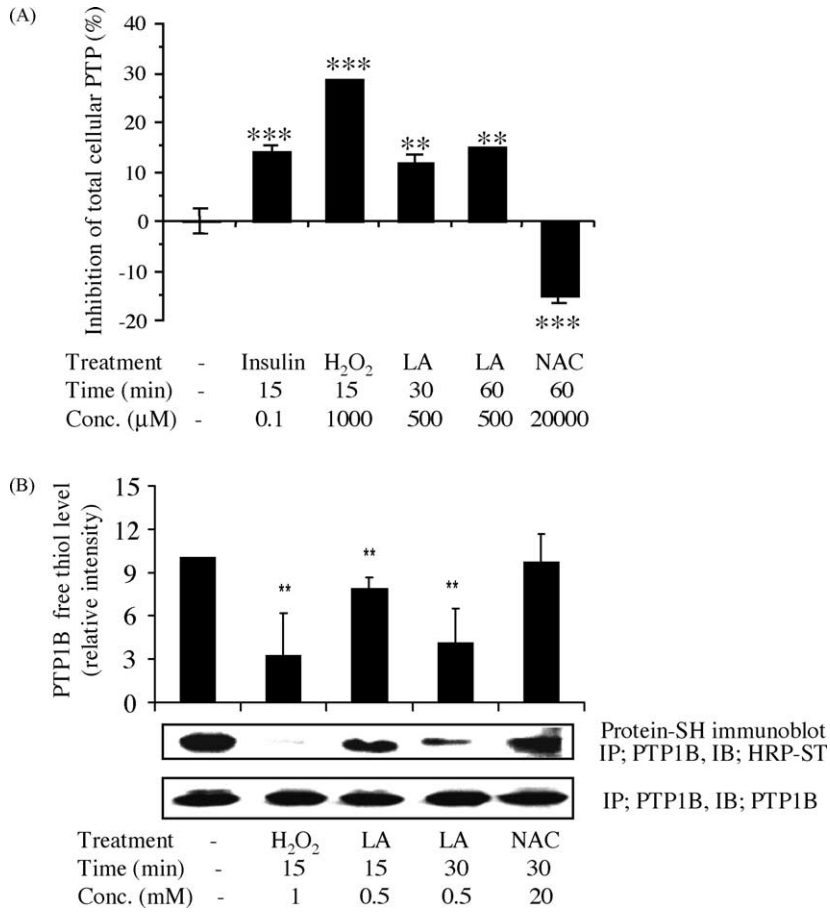

Fig. 8. Effect of α-lipoic acid on total protein tyrosine phosphatase activity and thiol reactivity of protein tyrosine phosphatase 1B. (A) 3T3-L1 adipocytes were treated with insulin, H_2O_2, α-lipoic acid (LA) or *N*-acetyl-L-cysteine (NAC) for times indicated. The total PTP activity was expressed as percent inhibition compared to the basal activity; ** or *** is significant at $P < 0.01$ or $P < 0.001$, respectively, compared to the basal. (B) Cells were treated with H_2O_2, α-lipoic acid (LA), or *N*-acetyl-L-cysteine (NAC) for times indicated. MBB was used to determine the level of thiol-biotinylated PTP1B. Each bar represents mean ± SD of three independent experiments. Band intensities were expressed relative to the intensity of control band, which was set at 10.

effect of α-lipoic acid (500 μM) with 100 nM of insulin was observed in 3T3-L1 adipocytes. This might be due to different cell types and/or concentrations of α-lipoic acid used in those studies. The lack of additivity and the wortmannin sensitivity of α-lipoic acid-stimulated glucose uptake indicate that α-lipoic acid, at least in part, exploits the same signaling pathway as insulin, to increase glucose uptake into 3T3-L1 adipocytes. In undifferentiated 3T3-L1 cells, the number of IR, the potency of post-receptor signaling events, and the magnitude of the biological responses to insulin are known to be dramatically lower [27–29]. Consistently, there was no significant change before differentiation between the basal and the insulin-stimulated glucose uptake. Although α-lipoic acid stimulation of glucose uptake in undifferentiated 3T3-L1 cells was far less than that observed in fully differentiated adipocytes, nevertheless, it was significant and sensitive to inhibition by wortmannin, suggesting that α-lipoic acid might more efficiently activate existing IR and/or post-receptor signaling events in undifferentiated cells.

The evidence now indicates that redox state of IR is involved in activation of the IR [13,30]. Reduced cysteine residues present in IR β-subunit were suggested to prevent spontaneous autophosphorylation of IR, whereas their oxidative modification was proposed to activate both tyrosine kinase domains [13]. In this study, α-lipoic acid as well as insulin and H_2O_2 decreased the level of reduced cysteine residues in IR β-subunit, by increasing intracellular oxidant level. Recently, α-lipoic acid was also demonstrated to inhibit glycogen synthesis via its oxidative activity and the uncoupling of mitochondria in rat soleus muscle [31]. α-Lipoic acid increased activity of the redox-sensitive transcription factor NF-κB both in 3T3-L1 adipocytes and in pre-adipocytes, suggesting its pro-oxidant effect (unpublished results). Long-term (24–48 hr) treatment of α-lipoic acid is known to increase GSH levels in a variety of cells including 3T3-L1 adipocytes [18,32]. However, in our study, short-term (0–2 hr) treatment of 3T3-L1 adipocytes with α-lipoic acid did not affect cellular GSH levels. α-Lipoic acid was also negligibly reduced to DHLA at this period of time (less than 1 μM DHLA was detected after 30 min incubation of cells with 250 μM α-lipoic acid; data not shown). These findings indicate that in 3T3-L1 adipocytes, α-lipoic acid shifts intracellular redox status toward an oxidizing condition, which may lead to oxidation of IR thiol groups and its activation. However, in our previous report [18], α-lipoic acid facilitated IR autophosphorylation *in vitro*, indicating direct interaction of oxidized α-lipoic acid with the thiols of the IR. Furthermore, although insulin-induced oxidation of IR thiol residue was less than H_2O_2- or α-lipoic acid-induced oxidation, insulin was able to increase IR tyrosine phosphorylation much stronger than the others in the same condition, suggesting that the oxidation of IR thiol groups may not be the only determining factor of IR tyrosine phosphorylation.

4.2. Role of intra/extracellular thiols on α-lipoic acid- or insulin-stimulated glucose uptake

NEM is known to inhibit insulin-stimulated glucose uptake into fat cells [33]. Several studies have investigated role of free thiol groups of the IR on *in vitro* autophosphorylation activity. Treatment of purified IR preparations with NEM after insulin stimulation inhibited autophosphorylation of the IR [34,35]. In contrast, pretreatment of isolated plasma membranes of 3T3-F442A adipocytes with NEM increased basal and insulin-stimulated IR autophosphorylation [36]. Consistently, pretreatment of 3T3-L1 adipocytes with NEM or PAO did not prevent insulin- or α-lipoic acid-stimulated IR autophosphorylation of intact cells but instead led to an increase in basal tyrosine phosphorylation of IR and other cellular proteins (data not shown). These findings indicate that thiol groups involved in IR autophosphorylation might not be accessible to large molecules such as NEM or PAO in intact cells. Nevertheless, the complete inhibition of α-lipoic acid- or insulin-stimulated glucose uptake demonstrates that NEM or PAO-sensitive component(s) of the insulin signaling pathway are probably downstream to the IR.

Although the putative site(s) of NEM action is not known, PAO was proposed to inhibit glucose uptake by increasing insulin-dependent Glut4 degradation [37], attenuating Glut4 recycling [38], and/or inhibiting PI3-K activity [39]. PAO was also found to inhibit PTP, suggesting that the increased basal tyrosine phosphorylation of cellular proteins might be due to an inhibitory effect of PAO on PTP [40,41]. Little is known about the role of extracellular thiol groups in glucose uptake. Alkylation of extracellular thiols by *p*CMPS did not affect autophosphorylation of IR elicited by α-lipoic acid or insulin. Whether the observed potentiation of α-lipoic acid-stimulated glucose uptake in *p*CMPS-treated cells is due to thiol modification of protein(s) involved in the regulation of glucose uptake or the enhanced local concentrations of α-lipoic acid at target site(s) remains to be elucidated. Nevertheless, these findings indicate that intracellular, rather than extracellular thiol groups have an important role in activation of the insulin signaling pathway and glucose uptake into 3T3-L1 adipocytes.

4.3. Role of NAPDH oxidase in α-lipoic acid- or insulin-stimulated glucose uptake

The biochemical properties of plasma membrane NADPH-dependent oxidase in fat cells have been partially characterized. Similar to the phagocyte enzyme, the adipocyte oxidase displays a several fold preference for NADPH over NADH and requires a flavin nucleotide for optimal activity. In contrast to the phagocyte system, DPI has no effect on basal or insulin-stimulated rates of NADPH-dependent H_2O_2 generation [42]. In contrast, DPI has recently been reported to inhibit insulin-stimulated H_2O_2 generation, IR autophosphorylation, and glucose

uptake into 3T3-L1 adipocytes [16]. In our study, however, DPI effectively inhibited both α-lipoic acid- or insulin-stimulated glucose uptake and IR autophosphorylation, while it marginally affected α-lipoic acid- or insulin-stimulated oxidant production. The underlying reason for the observed discrepancies related to the effect of DPI on insulin-stimulated oxidant production is not known. Nevertheless, the fact that DPI inhibited IR activation without attenuating the oxidant production suggests that other unidentified signaling component(s) may function in the interface of NADPH oxidase and IR. A thiol-reactive membrane-associated protein termed molecule X has recently been reported to covalently cross-link human IR β-subunit, though its function is not known [43].

In contrast to its inhibitory effect on glucose uptake, AV failed to prevent insulin-stimulated IR phosphorylation and slightly inhibited α-lipoic acid-induced IR phosphorylation, indicating that it may interfere with a component of the insulin signaling pathway downstream of the IR. PAO and NEM have also been reported to inhibit phagocyte NADPH oxidase by different mechanisms of action. PAO [44] inhibits activation of the phagocyte oxidase by binding to the β-subunit of flavo-cytochrome *b* in its resting state, whereas NEM [45] inhibits translocation and assembly of oxidase subunits to the plasma membrane during phagocyte activation. However, neither NEM nor PAO inhibited α-lipoic acid- or insulin-stimulated IR autophosphorylation. Whether differential effects of AV, NEM, PAO and DPI are due to differences in structural or biochemical properties of the adipocyte oxidase as compared to the phagocyte oxidase remains to be elucidated.

4.4. Inhibition of cellular PTP activity by α-lipoic acid or insulin

PTPs have in common a conserved 230-amino acid domain containing a cysteine residue that can catalyse the hydrolysis of protein phosphotyrosine residues by the formation of a cysteinyl-phosphate intermediate [46]. Considerable evidence indicates that ROS, including H_2O_2, oxidize and inactivate PTPs *in vivo* [47]. Insulin was also shown to inhibit cellular PTP activity and the specific activity of PTP1B by increasing H_2O_2 production in 3T3-L1 adipocytes [16]. Consistently, pretreatment of 3T3-L1 adipocytes with insulin, α-lipoic acid, or H_2O_2 inhibited total PTP activity whereas NAC, a thiol antioxidant, increased total PTP activity. α-Lipoic acid or H_2O_2 treatment of the cells also decreased level of thiol-biotinylated PTP1B, a tyrosine phosphatase that is strongly implicated in the down-regulation of insulin signaling *in vivo* [48]. These findings suggest that α-lipoic acid might modify the catalytic cysteine residue in PTPs resulting in their inactivation and thereby enhancing the early tyrosine phosphorylation cascade of the insulin signaling pathway.

The antioxidant activity of α-lipoic acid is considered to contribute to its beneficial effects in the treatment of diabetes. However, our findings now demonstrate that oxidants produced by α-lipoic acid are involved in activation of insulin receptor and in inactivation of protein tyrosine phosphatases leading to elevated glucose uptake into 3T3-L1 adipocytes. These findings indicate that the pro-oxidant effects of α-lipoic acid in tissues such as adipose tissue with a relatively low capacity to reduce α-lipoic acid may also contribute to the glucose lowering effects of α-lipoic acid. Further studies are warranted to evaluate the importance of alteration of intracellular redox status by α-lipoic acid in controlling glucose metabolism in animal models of diabetes as well as diabetic patients.

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