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α-Lipoic Acid Inhibits Adipocyte Differentiation by Regulating Pro-adipogenic Transcription Factors via Mitogen-activated Protein Kinase Pathways*

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Obesity is associated with a number of pathological disorders such as non-insulin-dependent diabetes, hypertension, hyperlipidemia, and cardiovascular diseases. α-Lipoic acid (LA) has been demonstrated to activate the insulin signaling pathway and to exert insulin-like actions in adipose and muscle cells. Based on this similarity LA is expected to promote adipogenesis in pre-adipocytes. Here, however, we report that LA inhibited differentiation of 3T3-L1 pre-adipocytes induced by a hormonal mixture or troglitazone. Northern blot analysis of cells demonstrated that this inhibition was accompanied with attenuated expression of adipocyte-specific fatty acid-binding protein and lipoprotein lipase. Electrophoretic mobility shift assay and Western blot analysis of cells demonstrated that LA modulates transcriptional activity and/or expression of a set of anti- or pro-adipogenic transcription factors. LA treatment of 3T3-L1 pre-adipocytes also resulted in prolonged activation of major mitogen-activated protein kinase signaling pathways but showed little or no effect on the activity of the insulin receptor/Akt signaling pathway. These findings suggest that LA inhibits insulin or the hormonal mixture-induced differentiation of 3T3-L1 pre-adipocytes by modulating activity and/or expression of pro- or anti-adipogenic transcription factors mainly through activating the MAPK pathways.

Obesity is closely correlated with the prevalence of diabetes and cardiovascular disease. Plasma levels of leptin, tumor necrosis factor (TNF)[1] α and non-esterified fatty acid are elevated in obesity and substantially contribute to the development of insulin resistance (1). Obesity is caused not only by hypertrophy of adipose tissue but also by adipose tissue hyperplasia, which triggers the transformation of pre-adipocytes into adipocytes (2).

The program of adipocyte differentiation is a complex process that involves coordinated expression of specific genes and proteins associated with each stage of differentiation. This process is regulated by several signaling pathways (3). Insulin, the major anabolic hormone, promotes *in vivo* accumulation of adipose tissue (4). Structurally unrelated inhibitors of phosphatidylinositol 3-kinase (PI3K), LY294002 and Wortmannin, were shown to block adipocyte differentiation in a time- and dose-dependent fashion (5), suggesting that the insulin receptor (IR)/Akt signaling pathway is important in transducing the pro-adipogenic effects of insulin. In contrast, mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) suppress the process of adipocyte differentiation (6, 7). TNFα is known to exert its anti-adipogenic effects, at least in part, through activation of the ERK pathway (6). However, p38 mitogen-activated protein kinase (p38K) is shown to promote adipocyte differentiation (8).

The signals that regulate adipogenesis either promote or block the cascade of transcription factors that coordinate the differentiation process. CCAAT element-binding proteins (C/EBP) β and δ and sterol response element-binding protein 1 (ADD1/SREBP1) are active during the early stages of the differentiation process and induce the expression and/or activity of the peroxisome proliferator-activated receptor γ (PPARγ), a pivotal coordinator of adipocyte differentiation. Activated PPARγ induces exit from the cell cycle, and in cooperation with C/EBPα, stimulates the expression of many metabolic genes such as glucose transporter-4, lipoprotein lipase (LPL) (9), and adipocyte-specific fatty acid-binding protein (aP2) (10), thus constituting a functional lipogenic adipocyte. JNK and ERK suppress this process by phosphorylating and thereby attenuating the transcriptional activity of PPARγ (6, 7). Besides these integral members of the adipogenesis program, other transcription factors such as AP-1 (11) and CREB (12) are known to promote adipogenesis, whereas nuclear factor-κB (NF-κB) suppresses adipocyte differentiation (13). Therefore, the activity and/or the expression of these transcription factors are attractive pharmacological targets for modulating adipocyte tissue formation and deposition.

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[1] The abbreviation used are: TNF, tumor necrosis factor; aP2, adipocyte-specific fatty acid-binding protein; AP-1, activator protein-1; C/EBP, CCAAT element-binding protein; CREB, cAMP-responsive element-binding protein; ERK, extracellular signal-regulated kinase; IR, insulin receptor; IRS, insulin receptor substrate; JNK, c-Jun N-terminal kinase; LA, α-lipoic acid; LPL, lipoprotein lipase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase; p38K, p38 mitogen-activated protein kinase; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR-responsive element; TZ, troglitazone; FBS, fetal bovine serum; PBS, phosphate-buffered saline.

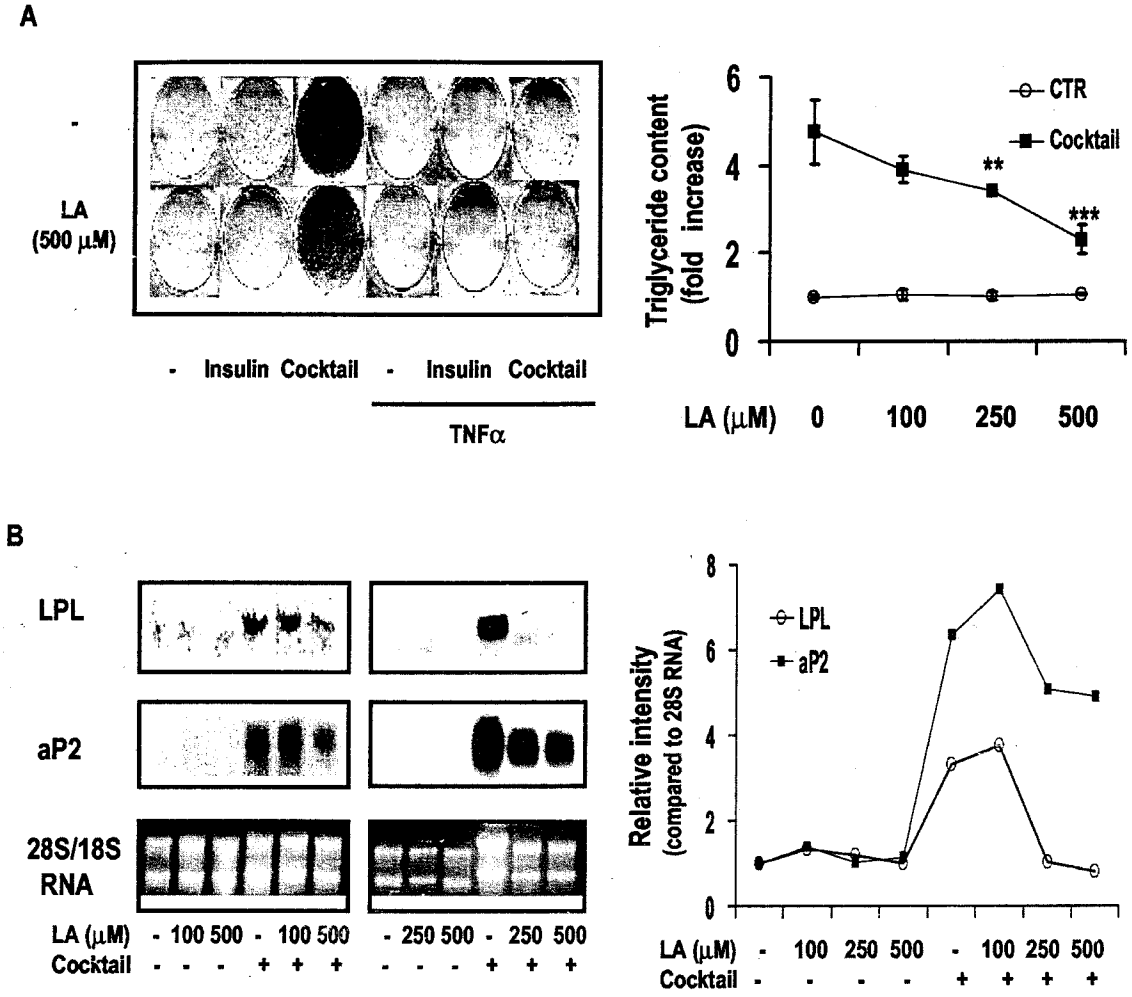

FIG. 1. **α-Lipoic acid inhibits pre-adipocyte differentiation induced by the hormonal mixture.** Post-confluent 3T3-L1 pre-adipocytes were treated with 10 nM insulin, 5 ng/ml TNFα, or the hormonal mixture (*Cocktail*) for 3 days in the absence or presence of indicated concentrations of LA. Cells were then treated with LA for additional 3 days in the normal medium. *A*, morphological changes associated with adipogenesis were photographed (*left panel*) based on staining cellular triglyceride deposition by Oil Red O, which was expressed as -fold increase compared with untreated cells (*CTR*, *right panel*). ** or ***, significant at $p < 0.01$ or $p < 0.001$ compared with the mixture-treated cells. *B*, mRNA levels of LPL and aP2 were determined by Northern blot analysis (*left panel*) and were expressed as -fold increase compared with 28 S rRNA (*right panel*).

Recent studies have demonstrated that LA facilitates glucose transport and utilization in fully differentiated adipocytes, as well as in animal models of diabetes (14–16). These insulin-like actions of LA were mainly mediated by activation of IR/Akt signaling pathway. Considering that insulin stimulates adipogenesis, this study was undertaken to investigate whether LA promotes differentiation of pre-adipocytes to mature adipocytes.

EXPERIMENTAL PROCEDURES

Reagents—Cell culture reagents including Dulbecco's modified Eagle's medium and fetal bovine serum (FBS) were purchased from Invitrogen. [α-^{32}P]dCTP and [γ-^{32}P]dATP were from PerkinElmer Life Sciences, and [*methyl*-^{3}H]thymidine was from Amersham Biosciences. 4G10 anti-phosphotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY). Protein A/G-agarose, polyclonal anti-IRβ subunit (C-19), and other antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). R form of α-lipoic acid (LA) was kindly provided by BASF AG (Ludwigshafen, Germany). If not specifically indicated, all other reagents were obtained from Sigma.

Cell Culture—3T3-L1 pre-adipocytes (American Type Culture Collection) were grown to confluence in Dulbecco's modified Eagle's medium containing 10% FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin as described previously (16). Two days after confluence (at a post-confluent stage), adipogenesis was induced by treating cells with a solution containing 5 μg/ml insulin, 0.25 μM dexamethasone, and 0.5 mM 3-isobutyl-1-methylxanthine, which is referred to as a "hormonal mixture." 20 mM stock solution of LA was freshly prepared in 20 mM HEPES, pH 7.4.

Oil Red O Staining—After the induction of differentiation, cells were stained with Oil Red O according to Ref. 17. Briefly, cells were washed twice with PBS and fixed with 10% formalin in PBS for 1 h; they were then washed three times with water. Cells were stained with Oil Red O (six parts of 0.6% Oil Red O dye in isopropanol and four parts of water) for 1 h. Excess of stain was removed by washing with water, and the stained cells were dried. The stained oil droplets was dissolved in isopropanol containing 4% Nonidet P-40 and were quantified by measuring the absorbance at 520 nm.

Northern Blotting—Total cellular RNA was purified from cultured cells using Trizol reagent (Molecular Research Center, Cincinnati, OH). RNA (10–30 μg) was electrophoresed on 1% agarose gel containing 37% formaldehyde and transferred to Hybond-N membrane (Amersham Biosciences) by capillary transfer. The membrane was fixed using an optimal UV cross-linking procedure and pre-hybridized at 68 °C in ExpressHyb hybridization solution (Clontech). cDNA probes for PPARγ, LPL, and aP2 were labeled with [α-^{32}P]dCTP (3000 Ci/mmol) using a random primer kit (TaKaRa). The blot was then washed twice with 2× SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) containing 0.05% SDS at 25 °C, 0.1× SSC containing 0.1% SDS at 55 °C and autoradiographed at −70 °C.

Immunoblotting—Cells in 100-mm dishes were washed with ice-cold PBS containing 1 mM Na_3VO_4 and lysed with a 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 mixture solution (Sigma). Cytosolic proteins were separated by 8–10% SDS-polyacrylamide gel, electrotransferred to a polyvinylidene difluoride membrane and immu-

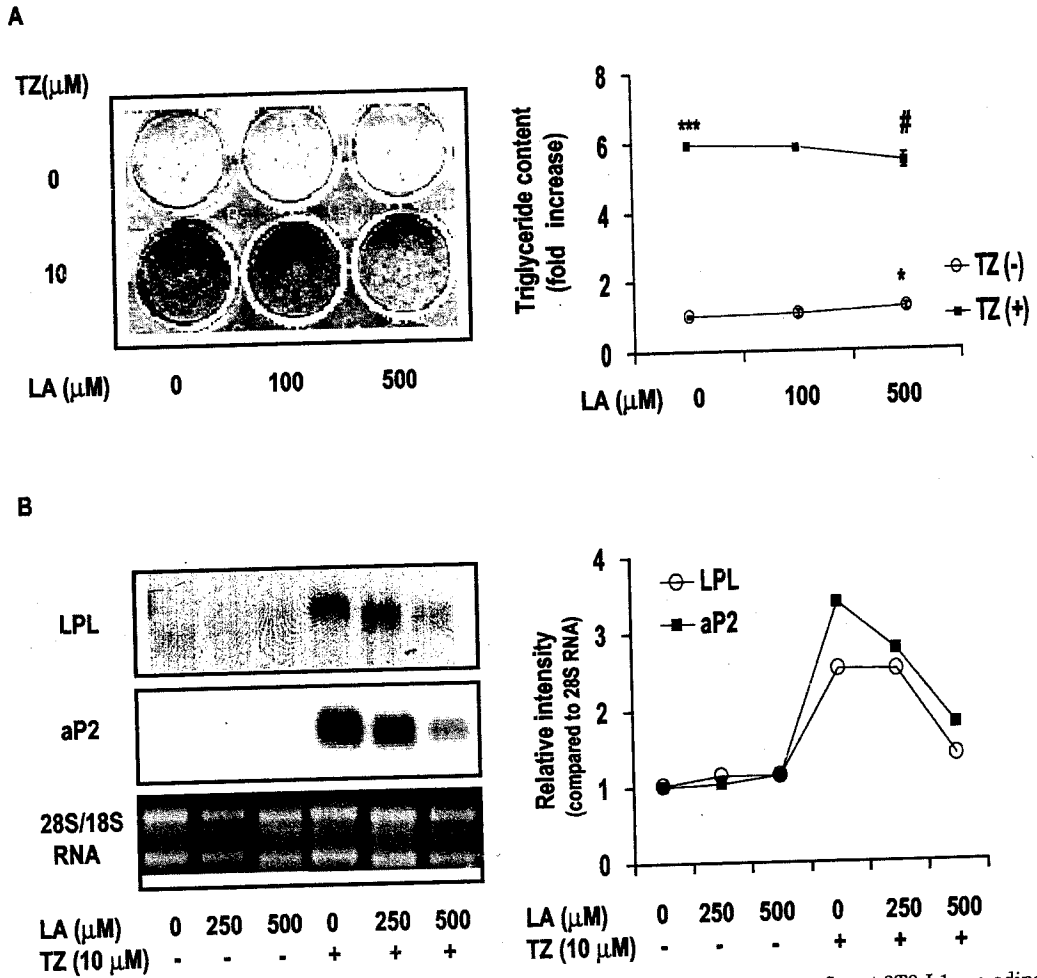

FIG. 2. **α-Lipoic acid suppresses troglitazone-induced pre-adipocyte differentiation.** Post-confluent 3T3-L1 pre-adipocytes were treated with 10 μM TZ for 6 days in the absence or presence of indicated concentrations of LA. The cells were then incubated in the normal medium for an additional 3 days. *A*, morphological changes associated with adipogenesis were photographed (*left panel*) based on staining cellular triglyceride deposition by Oil Red O, which was expressed as -fold increase compared with untreated cells (*right panel*). * or ***, significant at $p < 0.05$ or $p < 0.001$ compared with untreated cells. #, significant at $p < 0.05$ compared with TZ-treated cells. *B*, mRNA levels of LPL and aP2 were determined by Northern blot analysis (*left panel*) and were expressed as -fold increase compared with 28 S rRNA (*right panel*).

noblotted. The immunoreactive bands were visualized with an enhanced chemiluminescence reagent (Amersham Biosciences).

[^{3}H]Thymidine Uptake—Proliferation of the cells was evaluated by a [*methyl*-^{3}H]thymidine uptake. Post-confluent cells in 12-well culture plates were incubated with LA, insulin, and/or the hormonal mixture for 24 h. Eight h before harvest, 1 μCi of [*methyl*-^{3}H]thymidine was added to each well, and the incubation was stopped by washing the cells with ice-cold PBS containing 5% trichloroacetic acid. The [*methyl*-^{3}H]thymidine was quantitated using a liquid scintillation counter.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared as described previously (18). Cells were treated with LA, insulin, or the mixture for the indicated time period and lysed with a hypotonic buffer (10 mM Hepes, 1.5 mM $MgCl_2$, pH 7.5). The nuclei were pelleted by centrifugation at 3000 × *g* for 5 min and lysed in a hypertonic buffer (30 mM Hepes, 1.5 mM $MgCl_2$, 450 mM KCl, 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin). Following the lysis, the samples were centrifuged at 14,500 × *g* for 30 min, and the supernatant was retained for use in the DNA binding assay. Two double-stranded deoxyoligonucleotides corresponding to NF-κB, AP-1, CREB, Sp-1 (Promega), PPARγ, and C/EBPα (Santa Cruz) were end-labeled with [γ-^{32}P]dATP using T4 kinase (TaKaRa). The nuclear extracts were then incubated with 1 μg/μl poly(dI-dC) and ^{32}P-labeled DNA probe in a binding buffer (100 mM KCl, 30 mM Hepes, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml leupeptin) for 10 min. Thereafter, the reaction mixture was separated in 5% polyacrylamide gel. Following the electrophoresis, the gel was dried and subjected to autoradiography. The specificity of each probe was examined by the addition of 100-fold excess of each unlabeled probe. An electrophoretic mobility gel supershift assay was performed to discriminate PPARγ or C/EBPα from other isoforms by incubating the reaction mixture with 1 μg of anti-PPARγ or anti-C/EBPα antibody on ice for 30 min, respectively.

Transfection and Reporter Gene Assay—NIH-3T3 fibroblasts cultured on six-well tissue culture plates were transiently transfected with an expression plasmid for mouse PPARγ (1 μg/well) and a reporter plasmid with PPAR-responsive element (PPRE) (1 μg/well) using a LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's instructions. Twenty-four h after transfection, cells were supplemented with troglitazone, LA, or insulin, cultured for another 24–48 h, and then lysed in the recommended lysis buffer for luciferase assay. The luciferase activity was determined using the luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's instructions. (PPRE×3)-*tk*-luciferase and mouse PPARγ expression vectors were kindly provided by Dr. Young Yang (Korea Research Institute of Bioscience and Biotechnology).

Data Presentation—Data are presented as mean ± S.D. (or S.E.) of at least three independent experiments performed in triplicate. Differences between means were assessed by one-way analysis of variance. The minimum level of significance was set at $p < 0.05$.

RESULTS

α-Lipoic Acid Suppresses the Hormonal Mixture- or Troglitazone-induced Differentiation of 3T3-L1 Pre-adipocytes—3T3-L1 pre-adipocytes initiated their conversion to mature adipocytes 3 days after addition of either the hormonal mixture or insulin alone. Culturing these cells for another 6 days in the normal medium increased the number of fully differentiated adipocytes by 6.9 ± 0.1- or 2.3 ± 0.2-fold in cultures treated

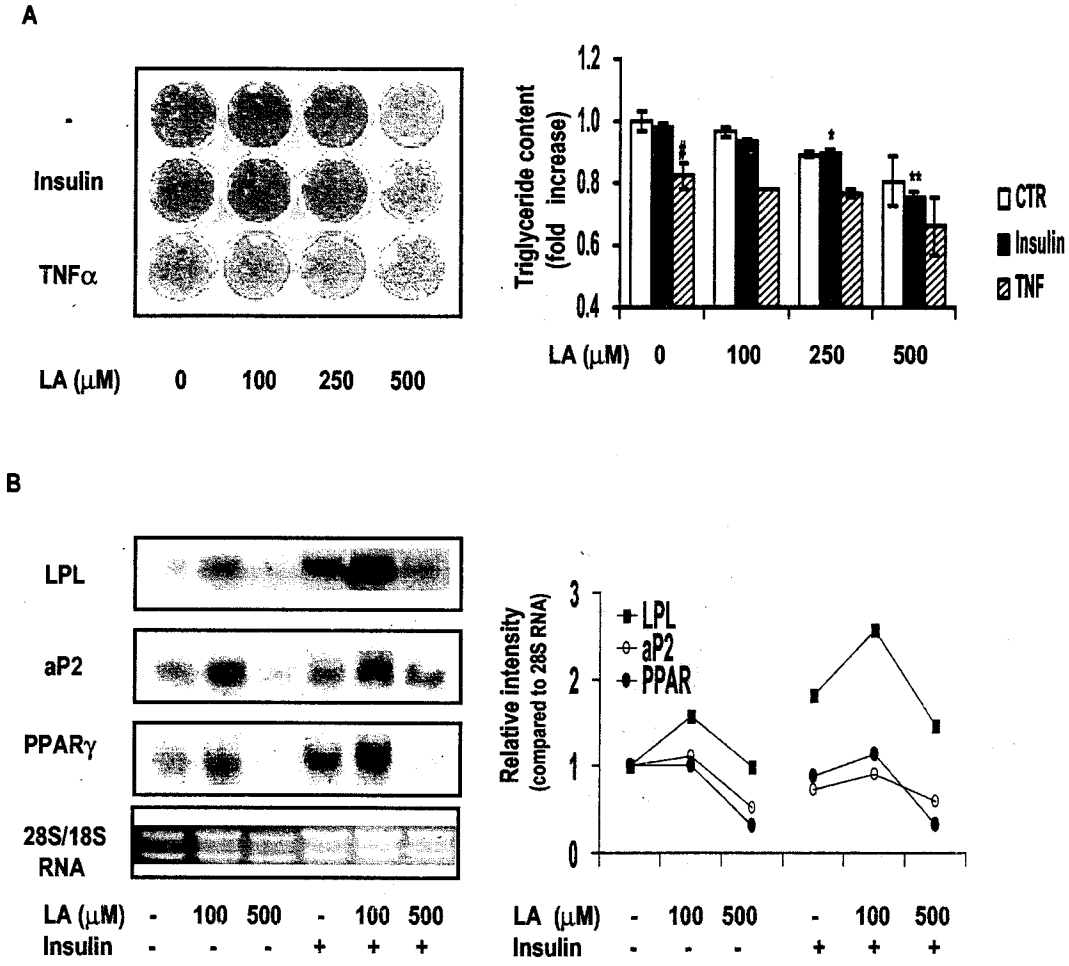

FIG. 3. **α-Lipoic acid promotes de-differentiation of adipocytes.** Adipogenesis was induced by treatment of 3T3-L1 pre-adipocytes with the hormonal mixture for 3 days. Thereafter, cells were treated with LA at indicated concentrations for 6 days in the absence or presence of 10 nM insulin or 5 ng/ml TNFα. *A*, morphological changes in the state of differentiation were photographed based on staining cellular triglyceride deposition by Oil Red O, which was expressed as -fold increase compared with untreated cells (*CTR, right panel*). * or **, significant at $p < 0.05$ or $p < 0.01$ compared with insulin-treated cells. #, significant at $p < 0.05$ compared with untreated cells. *B*, mRNA levels of LPL, aP2, and PPARγ were determined by Northern blot analysis (*left panel*) and were expressed as -fold increase compared with 28 S rRNA (*right panel*).

previously with the hormonal mixture or insulin alone, respectively (Fig. 1*A*). Treatment of cells with 5 ng/ml TNFα decreased differentiation by 13.2 ± 6.0%. However, co-treatment of the pre-adipocytes with LA inhibited the hormonal mixture- or insulin-induced differentiation and potentiated the anti-adipogenic effect of TNFα (Fig. 1*A*). Concordantly, mRNA levels of pro-adipogenic proteins such as aP2 and LPL were decreased by co-treatment with 250 or 500 μM LA and slightly increased by 100 μM LA (Fig. 1*B*). Troglitazone increases pre-adipocyte differentiation through up-regulation of PPARγ (19). In this study, 10 μM troglitazone increased adipocyte differentiation by 5.9 ± 0.1-fold. Co-treatment with 500 μM LA inhibited troglitazone-induced differentiation, which was accompanied with lower expression levels of aP2 and LPL genes (Fig. 2, *A* and *B*). These findings suggest that LA acts as a PPARγ antagonist.

α-Lipoic Acid Promotes De-differentiation of Adipocytes—Adipocytes can be reversibly de-differentiated in the presence of TNFα (20). Consistently, long-term (9 days) treatment of 3T3-L1 adipocytes with 5 ng/ml TNFα significantly de-differentiated the adipocytes by 17.7 ± 3.9%, whereas the presence of insulin maintained cells in the differentiated state. Post-treatment with 500 μM LA for 6 days subsequent to the induction of differentiation by the hormonal mixture promoted de-differentiation by 19.1 ± 8.3 or 23.2 ± 2.3% in the absence or presence of insulin, respectively. LA post-treatment also increased TNFα-induced de-differentiation by 19.6 ± 11.4% (Fig. 3*A*). Consistently, 6-day treatment with 500 μM LA after treatment with the hormonal mixture decreased mRNA levels of PPARγ, aP2, and LPL (Fig. 3*B*), indicating that the de-differentiating effect of LA was mediated by down-regulation of expression of these adipogenic factors. However, lower concentrations of LA (100 μM) did not de-differentiate adipocytes but slightly increased mRNA levels of the adipogenic factors, indicating a biphasic mode of LA action.

α-Lipoic Acid Regulates Insulin and Mitogen-activated Protein Kinase Signaling Pathways—LA is known to increase glucose uptake into fully differentiated 3T3-L1 adipocytes by activating the IR/Akt signaling pathway (16, 21). In pre-adipocytes, however, LA did not phosphorylate IR and IRS-1 within 30 min, whereas insulin strongly increased phosphorylation of both IR and IRS-1 from 5 min (Fig. 4, *A* and *B*) and lasted up to 4 h (data not shown). Moreover, LA transiently activated Akt whereas the insulin-induced Akt activation lasted up to several hours (Fig. 4*C*). Treatment of pre-adipocytes with 10 nM insulin or 500 μM LA also activated ERK, JNK, and p38K (Fig. 5, *A–C*). Although LA activated major MAPKs for longer than 30 min, insulin transiently activated them (~5 min). PD98059, an MAPK/ERK kinase kinase inhibitor, or Wortmannin, a PI3K inhibitor, abolished the phosphorylation of ERK or Akt, respectively, demonstrating that ERK or Akt activation was dependent on the

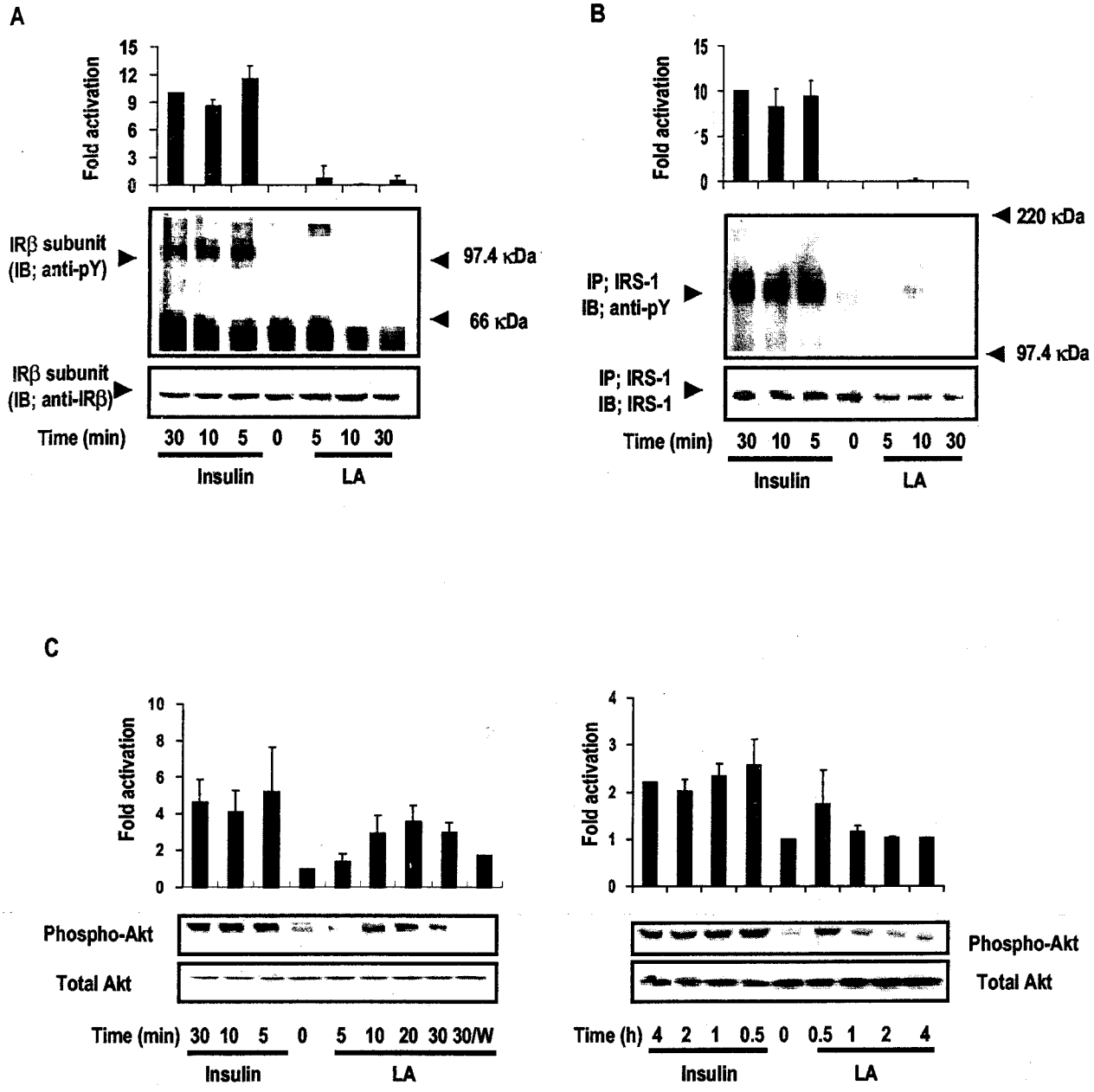

FIG. 4. **Effect of α-lipoic acid on insulin receptor/Akt signaling pathway in 3T3-L1 pre-adipocytes.** Post-confluent pre-adipocytes were serum-deprived for 4 h and pretreated with 400 nM wortmannin (*W*) for 30 min and treated with 10 nM insulin or 500 μM LA for the indicated times. Cells were washed with ice-cold PBS, and the phosphorylation states of the IR (*A*), IRS-1 (*B*), and Akt (*C*) were determined by Western blot analysis of the cell lysates (*A* and *C*) or immunoprecipitated (*IP*) IRS-1 (*B*). Each *bar* indicates mean ± S.E. of the -fold increases compared with the insulin-treated group (30 min), which was set at 10 (*A* and *B*) or to the untreated group (*C*). *IB*, immunoblot.

activity of their upstream kinase, MAPK/ERK kinase kinase, or PI3K, respectively.

In adipocytes at the early stage of differentiation, LA could not induce a detectable level of IR, IRS-1, and Akt phosphorylation within 60 min (Fig. 6, *A* and *B*), whereas LA strongly activated ERK and JNK (Fig. 6*C*) but not p38K (data not shown). Insulin, on the other hand, strongly activated IR, IRS, and Akt phosphorylation from 5 to 60 min, as well as ERK and JNK at the early stage of differentiation (Fig. 6).

α-Lipoic Acid Inhibits Insulin- or Hormonal Mixture-induced Mitotic Clonal Expansion—After growth arrest at confluence, pre-adipocytes re-enter the cell cycle and undergo several rounds of mitosis, referred to as mitotic clonal expansion (22). Initiation of the clonal expansion involves expression of immediate early genes, *fos*, *jun*, *myc*, C/EBPβ, and C/EBPδ, to drive confluent 3T3-L1 pre-adipocytes from G_0 into G_1 (23, 24). Insulin or the hormonal mixture promoted the clonal expansion 1 day after initiation of the induction as was evident by an increase in the rate of thymidine uptake (Fig. 7*A*). When cells were serum-deprived for 4 h, the protein expression of immediate early genes such as c-Fos and c-Jun was not detectable, whereas insulin or the hormonal mixture strongly induced expression of these proteins within several hours (Fig. 7*B*). LA co-treatment, however, significantly inhibited insulin- or the hormonal mixture-induced clonal expansion and decreased the expression of c-Fos and c-Jun (Fig. 7). Moreover, basal expression of c-Fos, c-Jun, and c-Myc in the presence of 10% FBS was diminished after treatment of cells with LA for 24 h in the absence or presence of insulin or the hormonal mixture (Fig. 7*C*). Two members of the retinoblastoma family of tumor suppressor proteins p107 and p130 are also known to regulate adipocyte differentiation by regulating its mitotic clonal expan-

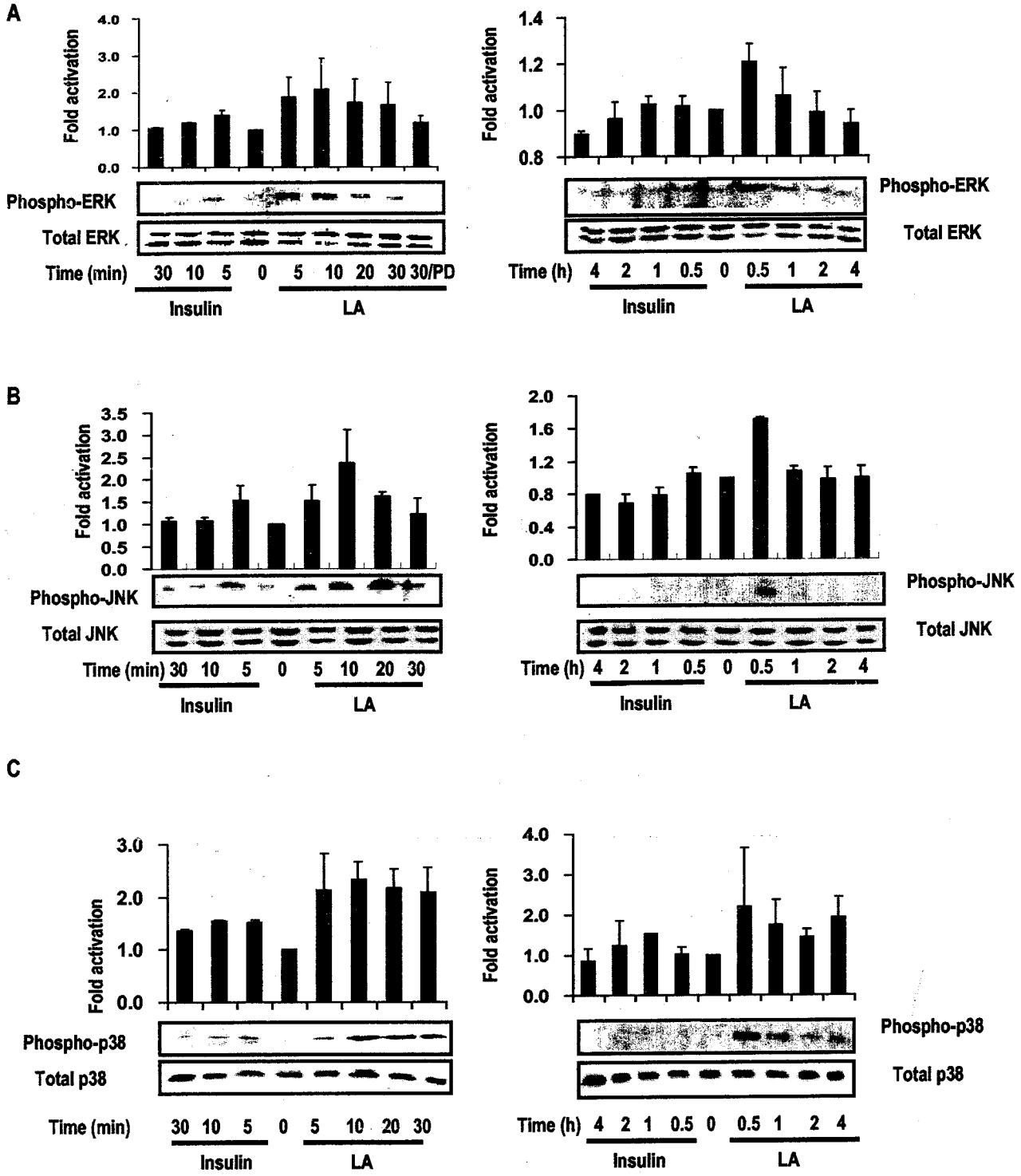

FIG. 5. **Effect of α-lipoic acid on mitogen-activated protein kinase signaling pathways in 3T3-L1 pre-adipocytes.** Post-confluent pre-adipocytes were serum-deprived for 4 h, when indicated, pretreated with 50 μM PD98059 for 30 min, and were then treated with 10 nM insulin or 500 μM LA for the indicated times. Cells were washed with ice-cold PBS, and phosphorylation states of ERK (*A*), JNK (*B*), and p38K (*C*) were determined by Western blot analysis of the cell lysates. Each *bar* indicates mean ± S.E. of the -fold increase compared with the untreated group.

sion phase. On a quiescent day 0 preadipocytes, high levels of p130 appear whereas p107 levels are barely detectable. After 24 h of the hormonal mixture stimulation, a significant increase appears in p107 levels with a concomitant decrease in the levels of p130 (25). In our study, p107, but not p130, is highly inducible by treatment with the hormonal mixture for 24 h, and LA co-treatment decreased the expression of p107 but not p130 (Fig. 7*C*). These findings suggest that LA inhibits the process of clonal expansion by suppressing the expression of several immediate early genes and some retinoblastoma family members.

Regulation of Pro- or Anti-adipogenic Transcription Factor Activities by α-Lipoic Acid—Pre-adipocytes displayed strong NF-κB activity, whereas fully differentiated adipocytes exhibited strong PPARγ and C/EBPα activities, regardless of the presence of FBS in their culture media (Fig. 8*A*). Fully differentiated adipocytes also displayed higher AP-1 and CREB activities compared with pre-adipocytes, which was further in-

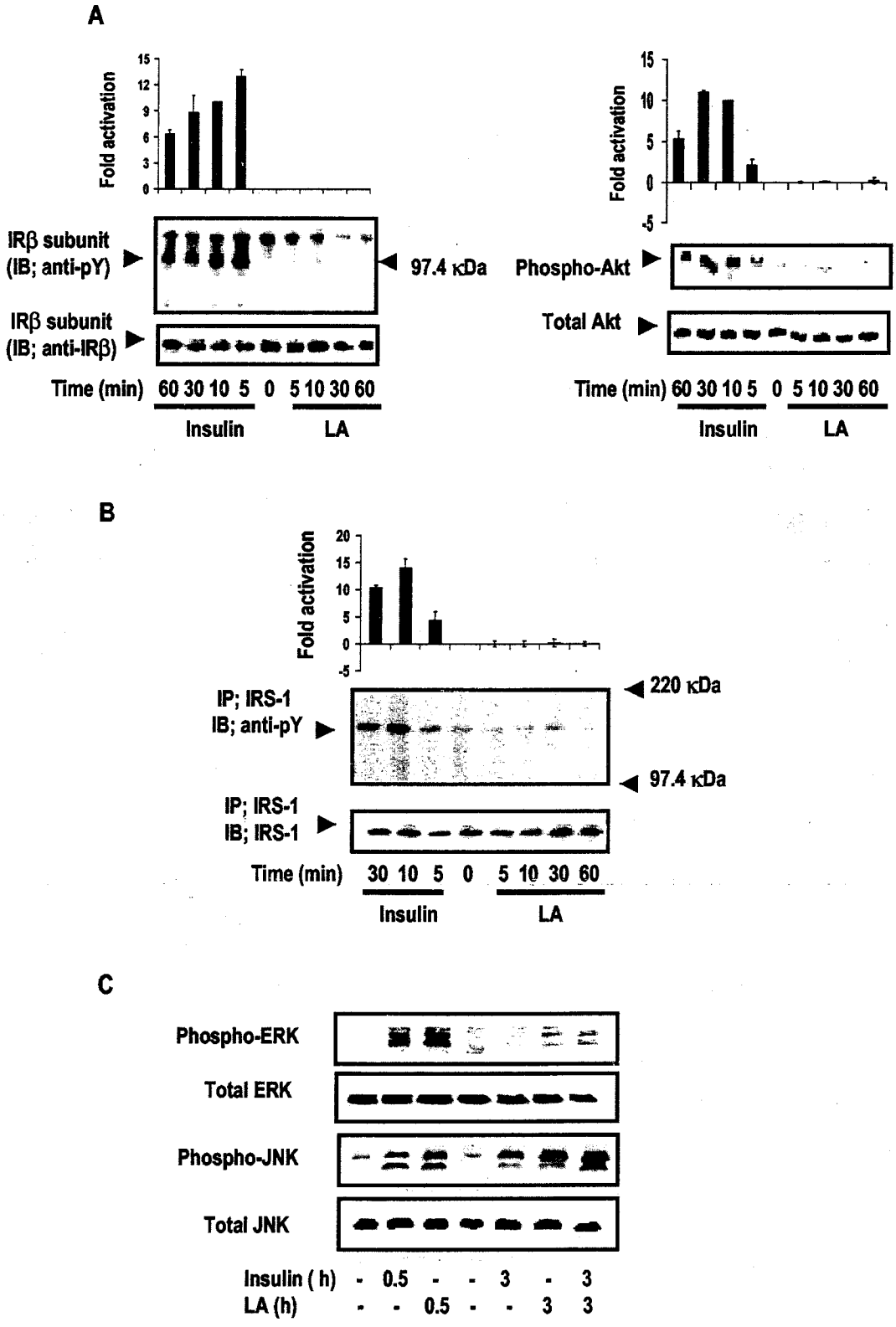

FIG. 6. **Effect of α-lipoic acid on insulin receptor/Akt signaling pathways (*A* and *B*) and mitogen-activated protein kinase (*C*) in adipocytes at the early stage of differentiation.** Adipogenesis was induced by treatment of 3T3-L1 pre-adipocytes with the hormonal mixture for 3 days. The cells were maintained in the normal medium for another 3 days. Cells were then serum-deprived for 4 h and were treated with 10 nM insulin or 500 μM LA for the indicated times. Cells were washed with ice-cold PBS, and phosphorylation states of indicated signaling proteins were determined by Western blot analysis of the cell lysates (*A* and *C*) or immunoprecipitates (*IP*) (*B*). Each *bar* indicates mean ± S.E. of the -fold increases compared with the insulin-treated group (10 min (*A*) or 30 min (*B*)), which was set at 10. *IB*, immunoblot.

creased in the presence of 10% FBS in the culture media. Sp-1 activity, however, was the same in all groups.

Two-h treatment of pre-adipocytes with LA strongly inhibited AP-1, C/EBP, and CREB activities, while increasing NF-κB activity; however, the hormonal mixture induced an opposite response (Fig. 8*B*). Interestingly, insulin alone also increased NF-κB activity, along with AP-1, C/EBP, and CREB activities. Co-treatment of pre-adipocytes with LA and insulin or the hor-

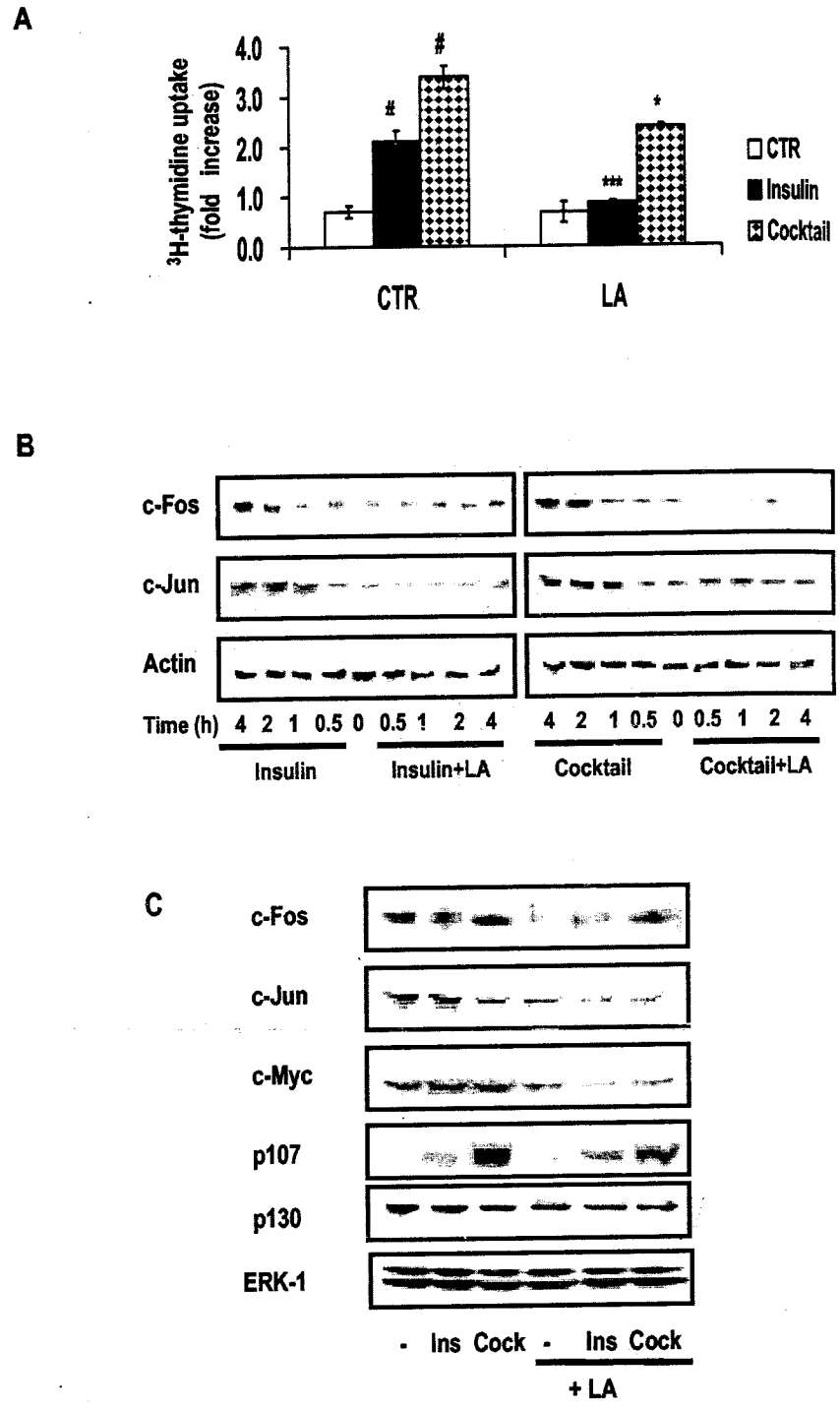

FIG. 7. **α-Lipoic acid inhibits the clonal expansion induced by insulin or the hormonal mixture in 3T3-L1 pre-adipocytes.** *A*, [^{3}H]thymidine uptake. Post-confluent pre-adipocytes were treated with 10 nM insulin or the hormonal mixture (*Cocktail*) in the absence or presence of 500 μM LA for 24 h. Eight h before harvest, the cells were pulsed with [*methyl*-^{3}H]thymidine, and the rate of thymidine uptake was measured using a liquid scintillation counter. #, significant at $p < 0.05$ compared with untreated cells. * or ***, significant at $p < 0.05$ or $p < 0.001$ compared with the hormonal mixture (*Cocktail*) or insulin-treated group. *B*, post-confluent pre-adipocytes were serum-deprived for 4 h and then treated with the hormonal mixture (*Cocktail*) or 10 nM insulin in the absence or presence of 500 μM LA for the indicated times. Cells were washed with ice-cold PBS, and protein levels of c-Fos and c-Jun were determined by Western blot analysis of the cell lysates. *C*, post-confluent pre-adipocytes were treated with the hormonal mixture (*Cock*) or 10 nM insulin (*Ins*) in the absence or presence of 500 μM LA for 24 h in the presence of 10% FBS. Cells were then washed with ice-cold PBS, and protein levels of c-Fos, c-Jun, c-Myc, p107, and p130 were determined by Western blot analysis of the cell lysates. Actin (*B*) and ERK-1 (*C*) were used as loading controls. *CTR*, no treatment.

monal mixture resulted in increased NF-κB activity and decreased activities of AP-1, C/EBP, and CREB in the nuclear fractions of pre-adipocytes. These findings strongly suggest that LA inhibits insulin- or the hormonal mixture-induced adipocyte differentiation by oppositely regulating nuclear translocation of pro- and anti-adipogenic transcription factors.

In adipocytes at the early stage of differentiation, 3 h of treatment with LA strongly inhibited transcriptional activity of PPARγ, C/EBPα, and AP-1, but not Sp-1, in the absence or presence of insulin (Fig. 9*A*). Pretreatment of cells with PD98059, an ERK inhibitor, or SP600125, a JNK inhibitor, partially reversed inhibition of C/EBPα or PPARγ, C/EBPα, and AP-1, respectively (Fig. 9*B*). Specific DNA binding activity of PPARγ and C/EBPα was also demonstrated by the supershift assay using anti-PPARγ and anti-C/EBPα antibodies, respectively (Fig. 9*C*).

Regulation of PPARγ Transactivation by α-Lipoic Acid in NIH-3T3 Fibroblasts—To confirm that the inhibitory effect of LA on PPARγ DNA binding activity affects its transactivation capacity, NIH-3T3 cells were transiently transfected with mouse PPARγ gene and PPRE vector. 20 μM troglitazone, a well known PPARγ agonist, strongly stimulated PPARγ-mediated luciferase activity after 24 or 48 h by 3.5 ± 0.1- or 3.1 ± 0.2-fold, respectively (Fig. 10). Insulin also acted as a weak PPARγ agonist and increased the luciferase activity by 1.5 ± 0.1- or 1.3-fold after 24 or 48 h, respectively. LA alone, however, weakly increased luciferase activity when used at 500 μM for 24 h. On the contrary, co-treatment of cells with LA signifi-

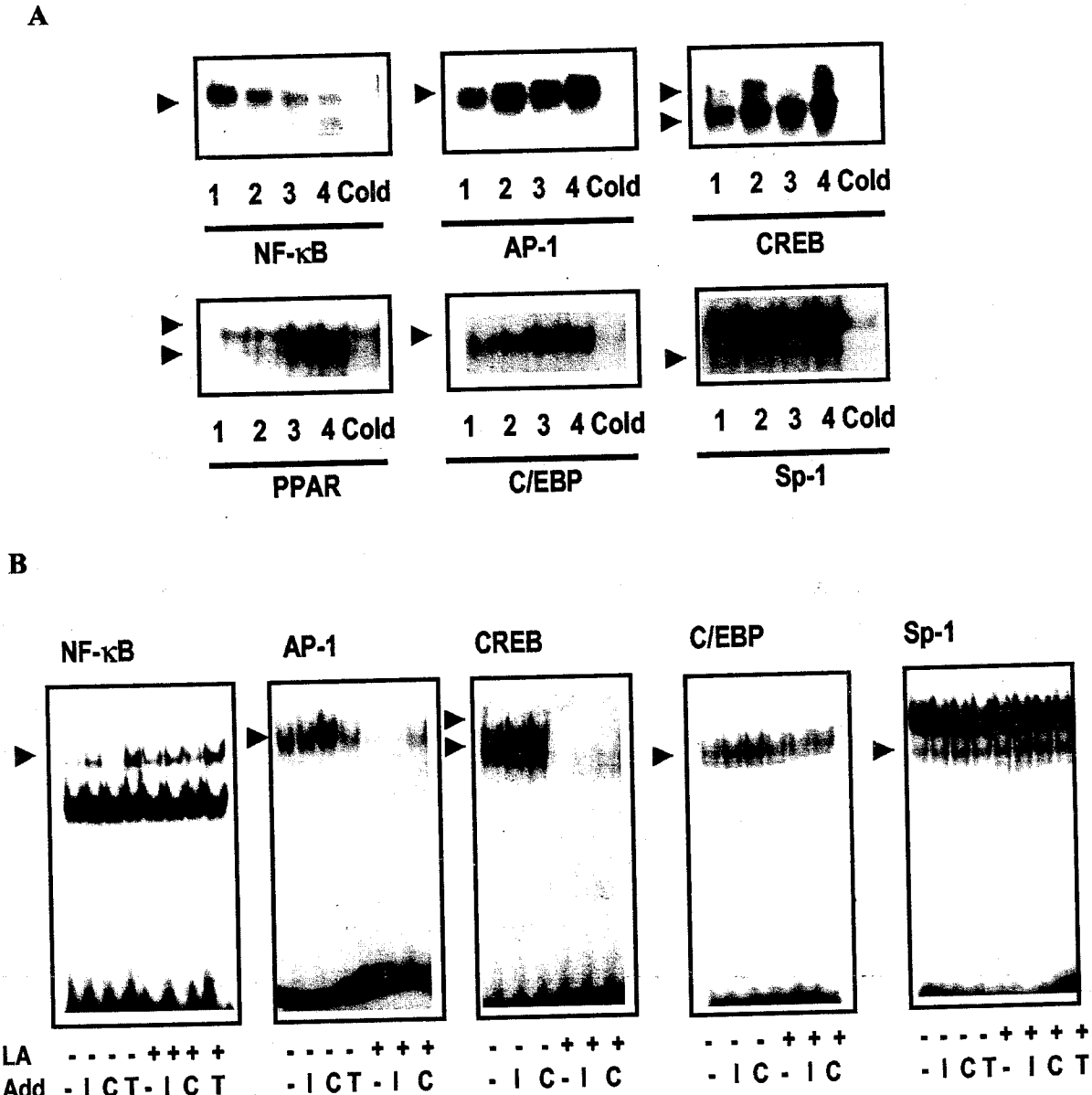

FIG. 8. **Regulation of anti- or pro-adipogenic transcription factors by α-lipoic acid in 3T3-L1 pre-adipocytes.** *A*, nuclear fractions prepared from 3T3-L1 pre-adipocytes (*1* and *2*) or fully differentiated 3T3-L1 adipocytes (*3* and *4*) maintained in cell culture media in the presence (*1* and *3*) or absence of FBS (*2* and *4*) were loaded into 5% polyacrylamide gel and probed by [γ-^{32}P]labeled NF-κB-, AP-1-, CREB-, PPAR-, C/EBP-, or Sp-1-binding sequences as described under "Experimental Procedures." The specificity of each probe was tested by addition of 100-fold excess of each cold probe. *B*, post-confluent pre-adipocytes were serum-deprived for 4 h and were treated with 10 nM insulin (*I*), the hormonal mixture (*C*), or 5 ng/ml TNFα (*T*) in the absence or presence of 500 μM LA for 2 h. Cells were then washed with ice-cold PBS, and the DNA binding activities of NF-κB, AP-1, CREB, and C/EBP were analyzed by electrophoretic mobility shift assay. *Arrows* indicate specific binding of nuclear proteins to the labeled DNA.

cantly inhibited troglitazone-induced PPARγ transactivation by 43.9 ± 0.9 or 19.5 ± 4.7% after 24 or 48 h, respectively. These findings demonstrate that LA alone acts as a weak PPARγ agonist, but it is a strong antagonist of PPARγ in the presence of other PPARγ agonists.

DISCUSSION

In this study, we demonstrated that LA inhibits differentiation of 3T3-L1 pre-adipocytes and that major MAPK signaling pathways mediate collateral actions of LA on clonal expansion and adipocyte maturation by attenuating the expression and activation of the immediate early genes such as c-Fos and c-Jun and by negatively regulating integral members of the differentiation program, PPARγ and C/EBPα.

MAPK Signaling Pathways Mediate Actions of α-Lipoic Acid on Adipogenesis—Several lines of evidence indicate that pro-adipogenic transcription factors such as PPARγ and members of the C/EBP family can be negatively regulated by MAPKs. Epidermal growth factor, platelet-derived growth factor, lipoxygenase-1 metabolites, and prostaglandin $F_2\alpha$ were shown to phosphorylate and attenuate transcriptional activity of PPARγ by activating MAPK signaling pathways (26–28). Similarly, LA treatment of pre-adipocytes inhibited insulin- or the hormonal mixture-induced transcriptional activity of PPARγ and C/EBPα, which was accompanied with strong activation of ERK and JNK. Furthermore, inhibitors of ERK or JNK activity abolished the inhibitory effect of LA on insulin- or the hormonal mixture-induced adipogenesis. On the other hand, LA hardly stimulated phosphorylation of IR or IRS-1 both in pre-adipocytes and in adipocytes at the early stage of differentiation. In particular, upon LA treatment, a transient Akt phosphorylation was detected in pre-adipocytes though it was not detectable in adipocytes at the early stage of differentiation. In

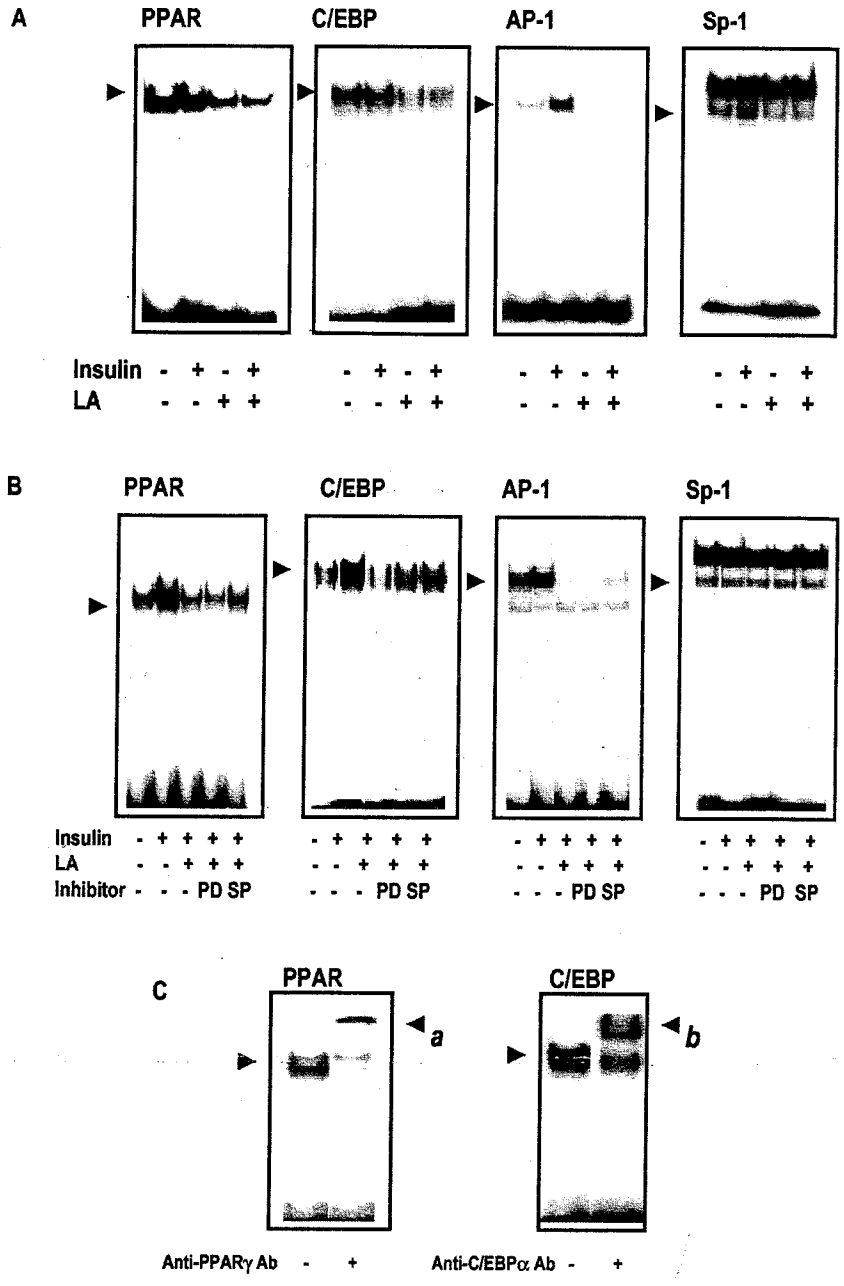

FIG. 9. **α-Lipoic acid regulation of pro-adipogenic transcription factors in adipocytes at the early stage of differentiation.** Adipogenesis was induced by treatment of 3T3-L1 pre-adipocytes with the hormonal mixture for 3 days after which cells were maintained in the normal medium for an additional 3 days. Thereafter, cells were serum-deprived for 4 h, were pre-treated with Me_2SO (as control), 50 μM PD98059 (*PD*), or 100 nM SP600125 (*SP*) for 30 min, and were then treated with 10 nM insulin and/or 500 μM LA for 3 h. Cells were washed with ice-cold PBS, and DNA binding activities of PPARγ, C/EBPα, AP-1, and Sp-1 were determined by electrophoretic mobility shift assay (*A* and *B*). Supershift assays for PPARγ and C/EBPα were conducted using anti-PPARγ and anti-C/EBPα antibodies. The supershifted complexes were indicated as *a* or *b*, respectively. *Arrows* indicate specific bindings of nuclear protein to the labeled DNA.

contrast, insulin strongly activated IR and IRS-1 and induced long lasting Akt activation in pre-adipocytes and in adipocytes at the early stage of differentiation. Taken together, these findings exclude possible involvement of Akt activation in LA-induced inhibition of adipogenesis and demonstrate that LA down-regulates PPARγ and C/EBPα through activation of MAPK signaling pathways. It should be emphasized that the underlying reason for the observation that LA activates the IR/Akt signaling pathway in fully differentiated adipocytes, but not in pre-adipocytes, might be the number of IRs and the potency of post-receptor signaling events that is known to be dramatically lower in pre-adipocytes.

Modulation of Auxiliary Transcription Factors in Adipogenesis by α-Lipoic Acid—Transcriptional activities of AP-1 and CREB were increased in fully differentiated 3T3-L1 adipocytes, as well as after 2 h of treatment with the hormonal mixture in 3T3-L1 pre-adipocytes. AP-1 is involved in transcriptional regulation of aP2 and LPL genes (29, 30). CREB appears to stimulate transcription of several adipocyte-specific genes such as aP2, fatty acid synthetase, and phosphoenolpyruvate carboxykinase (12). LA, however, strongly down-regulated AP-1 and CREB activities whereas it up-regulated NF-κB activity in pre-adipocytes. Many anti-adipogenic factors such as proinflammatory cytokines (31), TNFα (13), and endrin (17) are also known to up-regulate NF-κB activity, whereas pro-adipogenic factors such as troglitazone display an opposite effect in 3T3-L1 cells (32). Considering that AP-1 (33), CREB (34), and NF-κB (35) mediate major downstream effects of MAPK signaling pathways, our findings suggest that LA activation of the MAPK signaling pathways leads to the differential regulation of these transcription factors, which eventually results in decreased expression of the adipocyte-specific genes, consequently contributing to the suppression of adipogenesis.

MAPK Signaling Pathways Mediate Actions of α-Lipoic Acid on Cell Cycle and Clonal Expansion—In the course of adipogenesis, one of the first events that occur following hormonal induction is re-entry of growth-arrested pre-adipocytes into the cell cycle. In this study, LA was demonstrated to inhibit the process of clonal expansion when induced by insulin or the hormonal mixture, indicating that insulin and LA oppositely

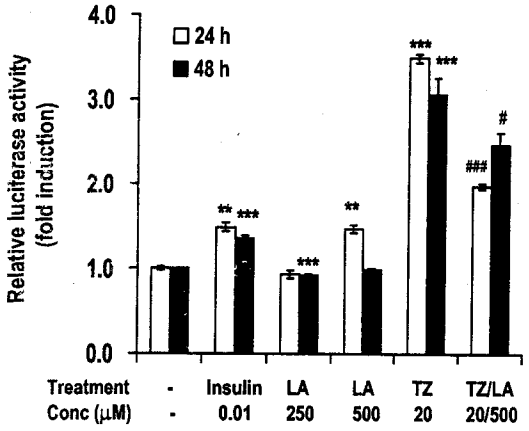

FIG. 10. **α-Lipoic acid inhibits troglitazone-induced PPARγ transactivation in PPARγ-transfected NIH-3T3 fibroblasts.** Cells in 6-well plates were co-transfected with mouse PPARγ expression vector and PPRE-*tk*-luciferase vector using LipofectAMINE 2000 (Invitrogen) as described under "Experimental Procedures." Twenty-four h later, cells were treated with 10 nM insulin, 250 or 500 μM LA, and 20 μM TZ for another 24 or 48 h. The luciferase activity was then measured using a luciferase assay kit (Promega). The relative activity was expressed as -fold increase compared with untreated cells. ** or ***, significant at $p < 0.01$ or $p < 0.001$ compared with untreated cells. # or ###, significant at $p < 0.05$ or $p < 0.001$ compared with TZ-treated cells at each *time point*.

regulate cell cycle progression. This differential effect seems to be because of the potency and/or the kinetics of activating of MAPK and IR/Akt signaling pathways. Both insulin and LA activated MAPK signaling pathways in pre-adipocytes. However, insulin, but not LA, also strongly activated the IR/Akt signaling pathway. This observation indicates that progression in the cell cycle and clonal expansion may require activation of both MAPK and IR/Akt signaling pathways. On the other hand, insulin-induced MAPK activation was transient whereas that of LA lasted longer, indicating that duration of MAPK activation might be another important factor in determining the fate of a cell in the cell cycle. Indeed, transient activation of MAPK has been considered as a contributor to cell cycle progression whereas its prolonged activation can result in cell cycle arrest via induction of $p21^{Cip1/Waf1}$ expression and inhibition of cyclin-dependent kinase activity (36, 37). It should be emphasized that JNK is known to activate p53, which triggers activation of several proteins involved in cell cycle arrest such as $p21^{Cip1/Waf1}$ (38). This evidence supports the notion that activation of MAPKs mediates the inhibitory effect of LA on the clonal expansion process by suppressing the expression of the immediate early genes.

Conclusions—Several PPARγ agonists such as thiazolidinediones have been recommended for the treatment of diabetes by improving insulin sensitivity and glucose uptake (19). Treatment with current PPARγ agonists, however, leads to increased adiposity and body weight gain in rodents (39), which subsequently contribute to the enhanced insulin resistance. In our study, LA at lower concentrations (100 μM) promoted adipogenesis whereas at higher concentrations (250 and 500 μM) it was inhibitory. Importantly, LA inhibited adipogenesis induced by insulin or troglitazone indicating that co-treatment with LA may be beneficial in preventing obesity induced by PPARγ agonists by maintaining optimal adipogenesis. Although LA increases glucose uptake into muscle or adipose tissue by activating the IR/Akt signaling pathway, the underlying mechanism for regulation of adipogenesis by LA appears to be different. Our findings strongly suggest that LA regulates adipogenesis mainly through activation of MAPKs such as ERK and JNK independent of activation of IR/Akt signaling pathway.

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