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Integrin-Linked Kinase Is Involved in TNF-α-Induced Inducible Nitric-Oxide Synthase Expression in Myoblasts

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ABSTRACT

Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine produced by activated macrophages. Nitric oxide (NO) is a highly reactive nitrogen radical implicated in inflammatory responses. We investigated the signaling pathway involved in inducible nitric oxide synthase (iNOS) expression and NO production stimulated by TNF-α in cultured myoblasts. TNF-α stimulation caused iNOS expression and NO production in myoblasts (G7 cells). TNF-α-mediated iNOS expression was attenuated by integrin-linked kinase (ILK) inhibitor (KP392) and siRNA. Pretreatment with Akt inhibitor, mammalian target of rapamycin (mTOR) inhibitor (rapamycin), NF-κB inhibitor (PDTC), and IκB protease inhibitor (TPCK) also inhibited the potentiating action of TNF-α. Stimulation of cells with TNF-α increased ILK kinase activity. TNF-α also increased the Akt and mTOR phosphorylation. TNF-α mediated an increase of NF-κB-specific DNA–protein complex formation, p65 translocation into nucleus, NF-κB-luciferase activity was inhibited by KP392, Akt inhibitor, and rapamycin. Our results suggest that TNF-α increased iNOS expression and NO production in myoblasts via the ILK/Akt/mTOR and NF-κB signaling pathway. J. Cell. Biochem. 109: 1244–1253, 2010.

KEY WORDS: ILK; TNF-α; iNOS; NF-κB; MYOBLASTS

Muscle loss occurs after injury as a result of aging and in a variety of cachectic conditions associated with severe disease states such as AIDS, cancer, and congestive heart failure. A change in lean muscle mass is often attributed to a changed balance between protein synthesis and degradation rates in the myofibers [Mitch and Goldberg, 1996]. On the other hand, muscle loss or reduced muscle growth may be related to reduced capacity of regeneration from satellite cells. Regulation of either of these biological processes involves complex interactions among multiple growth factors and cytokines [Brink et al., 2002]. Tumor necrosis factor (TNF)-α is produced from macrophages and other cells [Bastos et al., 2009]. It has been also reported that TNF-α is associated with

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advanced stage of disease of different tumor [Balkwill, 2009]. TNF-α-mediated effects by two receptors that can induce apoptosis as well as proliferation in different cell lines [Jurisic et al., 2006]. TNF-α, originally known as cachectin, is considered to be a catabolic factor that mediates the development of skeletal muscle wasting. In patients, elevated circulating levels of this cytokine are generally associated with catabolic states [Argiles et al., 2005], and muscle wasting as a result of changed protein metabolism has been observed after acute or chronic administration of TNF-α [Goodman, 1991; Garcia-Martinez et al., 1993], although it was at the same time reported that no change in protein metabolism occurred in isolated muscle preparations [Goodman, 1991].

Nitric oxide (NO) is a highly reactive nitrogen radical implicated in multiple biological processes, including regulation of vascular tone, platelet and leukocyte adhesion, and neurotransmission, and mediation of excessive vasodilatation and cytotoxic actions of macrophages against microbes and tumor cells [Fitzpatrick et al., 2008; Weigert and Brune, 2008; Kanwar et al., 2009]. Its formation is regulated by a family of enzymes, known as nitric oxide synthase (NOS), which oxidize the guanidine moiety of L-arginine, resulting in the equimolar production of NO and L-citrulline. Two major classes of NOS have been described based on their expression and regulation. The constitutive form present in neurons (nNOS) or endothelial cells (eNOS) is a calcium-dependent enzyme. The inducible form (iNOS), on the other hand, present in macrophages and other cells, is regulated at the transcriptional level in response to lipopolysaccharide or certain proinflammatory cytokines and does not require calcium for its activity [Mori and Gotoh, 2000; Trajkovic, 2001].

Integrin-linked kinase (ILK), a potential candidate signaling molecule, has been shown to be capable of regulating integrin-mediated signaling [Hannigan et al., 1996]. ILK can interact with the cytoplasmic domain of β-integrin subunits and is activated by both integrin activation as well as growth factors and is an upstream regulator of Akt [Wu and Dedhar, 2001]. It has been reported that ILK is mediated iNOS and cyclooxygenase-2 (COX-2) expression in macrophage [Tan et al., 2002]. In addition, ILK also has involved in ultrasound-mediated iNOS expression in osteoblasts [Tang et al., 2007]. Whether ILK activation involved in TNF-α-induced iNOS expression and NO production are mostly unknown. In the present study, we explored the intracellular signaling pathway involved in TNF-α-induced iNOS expression and NO production in myoblastic cells. The results showed that TNF-α activates ILK/Akt/mammalian target of rapamycin (mTOR) and NF-κB pathway, leading to up-regulation of iNOS expression and NO production.

MATERIALS AND METHODS

MATERIALS

Protein A/G beads, anti-mouse and anti-rabbit IgG-conjugated horseradish peroxidase, rabbit polyclonal antibodies specific for α-tubulin, p-Akt, Akt, IκBα, p-IκBα, IKKα/β, p65, and the small interfering RNAs (siRNAs) against ILK and control for experiments using targeted siRNA transfection (each consists of a scrambled sequence that will not lead to the specific degradation of any known cellular mRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies specific for phospho-mTOR (Ser-2448), glycogen synthase kinase 3β (GSK3β), phospho-GSK3β, phospho-IKKα/β (Ser$^{180/181}$), and phospho-p65 (Ser^{536}) were purchased from Cell Signaling and Neuroscience (Danvers, MA). Rabbit polyclonal antibody for ILK and mTOR siRNA were purchased from Upstate Biotechnology (Lake Placid, NY). Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol-2-((R)-2-*O*-methyl-3-*O*-octadecylcarbonate)), rapamycin, pyrrolidine dithiocarbamate (PDTC), and TPCK were purchased from Calbiochem (San Diego, CA). KP-392 was purchased from Kinetek Pharmaceuticals (Vancouver, Canada). The NF-κB-luciferase plasmid was purchased from Stratagene (La Jolla, CA). The Akt (Akt K179A) dominant-negative mutant was a gift from Dr. W.M. Fu (National Taiwan University, Taipei, Taiwan). The iNOS promoter construct (piNOS-Luc) was a gift from Dr. E. A. Ratovitski (Johns Hopkins University). pSV-β-galactosidase vector and luciferase assay kit were purchased from Promega (Madison, MA). All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO).

CELL CULTURES

Murine myoblasts G7 cells were obtained from American Type Culture Collection (ATCC; Rockville, MD). Cells were cultured in DMEM supplemented with 100 IU/ml penicillin, 10 μg/ml streptomycin, and 50 μg/ml neomycin in the presence of 10% fetal bovine serum (FBS).

ASSAY OF NO

Cells (3×10^5 cells) were exposed to TNF-α. Production of NO was assayed by measuring the stable metabolite of nitrite levels in the culture medium. Sample aliquots (100 μl) were mixed with 100 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylenediamine dihydrochloride/2% phosphoric acid) in 96-well plate and incubated at 25°C for 10 min. The absorbance at 550 nm was measured on a microplate reader [Tang et al., 2007].

WESTERN BLOT ANALYSIS

The cellular lysates were prepared as described previously [Tan et al., 2009]. Proteins were resolved on SDS–PAGE and transferred to Immobilon polyvinyldifluoride (PVDF) membranes. The blots were blocked with 4% BSA for 1 h at room temperature and then probed with rabbit anti-human antibodies against p-Akt, Akt p-IKK, or IKK (1:1,000) for 1 h at room temperature. After three washes, the blots were subsequently incubated with a donkey anti-rabbit peroxidase-conjugated secondary antibody (1:1,000) for 1 h at room temperature. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

ILK KINASE ASSAY

ILK enzymatic activity was assayed in G7 cells lysed in Nonidet P-40 buffer (0.5% sodiumdeoxycholate, 1% Nonidet P-40, 50 mM HEPES (pH 7.4), 150 mM NaCl) as previously reported [Tang et al., 2007]. Briefly, ILK was immunoprecipitated with ILK antibody overnight at 4°C from 250 μg of lysate. After immunoprecipitation, beads were resuspended in 30 μl of kinase buffer containing 1 μg of

recombinant substrate (GSK3β fusion protein) and 200 μM cold ATP, and the reaction was carried out for 30 min at 30°C. The phosphorylated substrate was visualized by Western blot with phospho-GSK3β antibody. Total GSK3β was detected with the appropriate antibody.

QUANTITATIVE REAL-TIME PCR

The quantitative real-time PCR (qPCR) analysis was carried out using Taqman® one-step PCR Master Mix (Applied Biosystems, Foster City, CA). One hundred nanograms of total cDNA were added per 25-μl reaction with sequence-specific primers and Taqman® probes. Sequences for all target gene primers and probes were purchased commercially (β-actin was used as internal control) (Applied Biosystems). Quantitative RT-PCR assays were carried out in triplicate on StepOnePlus sequence detection system. The cycling conditions were 10-min polymerase activation at 95°C followed by 40 cycles at 95°C for 15 s and 60°C for 60 s. The threshold was set above the nontemplate control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted as C_T).

TRANSFECTION AND REPORTER GENE ASSAY

Myoblasts were co-transfected with 0.8 μg iNOS-luciferase plasmid and 0.4 μg β-galactosidase expression vector. Cells were grown to 80% confluence in 12-well plates and were transfected on the following day by Lipofectamine 2000 (LF2000; Invitrogen). DNA and LF2000 were premixed for 20 min and then applied to the cells. After 24 h transfection, the cells were incubated with the indicated agents. After a further 24 h incubation, the media were removed, and cells were washed once with cold PBS. To prepare lysates, 100 μl reporter lysis buffer (Promega) was added to each well, and cells were scraped from dishes. The supernatant was collected after centrifugation at 13,000 rpm for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (20–30 μg) were placed into wells of an opaque black 96-well microplate. An equal volume of luciferase substrate was added to all samples, and luminescence was measured in a microplate luminometer. The value of luciferase activity was normalized to transfection efficiency monitored by the co-transfected β-galactosidase expression vector.

ELECTROPHORETIC MOBILITY SHIFT ASSAY

Electrophoretic mobility shift assay was performed by using EMSA "gel shift" kit (Panomics, Redwood City, CA) according to the manufacturer's protocol. NF-κB consensus ologonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was used. Nuclear extract (3 μg) of cells was incubated with poly d(I-C) at room temperature for 5 min. The nuclear extract was then incubated with biotin-labeled probes and the incubated at room temperature for 30 min. After electrophoresis on a 6% polyacrylamide gel, the samples on gel were transferred onto a presoaked Immobilon-Ny+ membrane (Millipore, Billerica, MA). The membrane was baked at 80°C for 1 h, cross-linked in an oven for 3 min and then developed by adding the blocking buffer and streptavidin–horseradish peroxidase conjugate and then subjected to Western blot analysis.

IMMUNOFLUOROCYTOCHEMISTRY

Cells were cultured in 12-mm coverslips. After treatment with TNF-α, cells were fixed with 4% paraformaldehyde at room temperature. Thirty minutes later, 4% nonfat milk in PBS containing 0.5% Triton X-100 was added to the cells. The cells were then incubated with rabbit anti-p65 (1:100) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (1:500; Leinco Technology, Inc., St. Louis, MO) for 1 h, respectively. The FITC was detected using a Zeiss fluorescence microscope.

STATISTICS

For statistical evaluation, Mann–Whitney *U*-test for non-Gaussian parameters and Student's *t*-test for Gaussian parameters (including Bonferroni correction). The difference is significant if the *P*-value is <0.05

RESULTS

ILK ACTIVATION IS MEDIATED TNF-α-INDUCED iNOS EXPRESSION IN MURINE MYOBLASTS

Murine myoblasts were chosen to investigate the signal pathways of TNF-α in iNOS expression, an inflammatory response gene. Treatment of myoblasts (G7 cells) with TNF-α (10 ng/ml) increased the protein expression of iNOS in a time-dependent manner (Fig. 1A). The effect of TNF-α on the generation of NO from G7 cells is shown in Figure 1B. Treatment of cells for 24 h with TNF-α also resulted in increased NO production in a dose-dependent manner. It has been reported that ILK plays an important role in iNOS

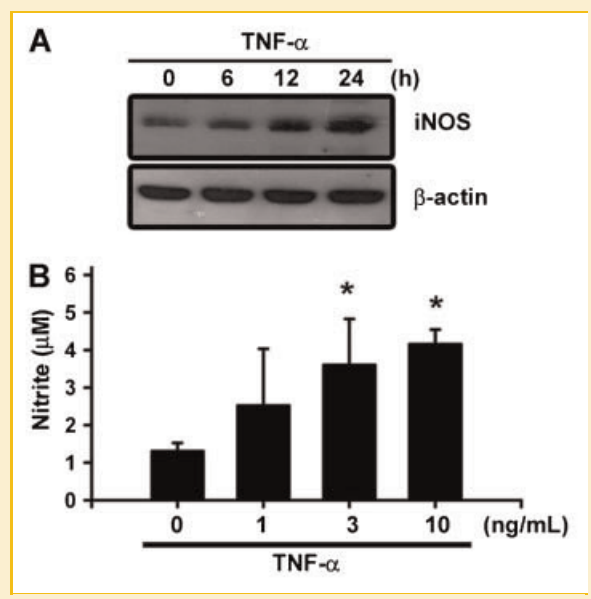

Fig. 1. TNF-α induces iNOS expression and NO production in cultured myoblasts. A: Cells were incubated with TNF-α (10 ng/ml) for 6, 12, and 24 h, and iNOS protein expression was measured by Western blot. B: Cells were incubated with TNF-α (1–10 ng/ml) for 24 h. The cultured media were collected. The production of NO was evaluated by Griess reaction by measuring the level of nitrite. Results are expressed of four independent experiments performed in triplicate. $^{*}P < 0.05$ as compared with basal level.

production in macrophage [Tan et al., 2002]. To explore whether ILK is involved in TNF-α-induced iNOS expression and NO production, ILK inhibitor KP-392 or ILK siRNA were used. As shown in Figure 2A–C, pretreatment of myoblasts with KP-392 inhibited TNF-α-induced iNOS expression and NO production. In addition, treatment of cells with KP392 (10 μM) did not affect cell viability, which was assessed by the MTT and TUNEL assay (Supplementary Fig. S2). Transfection of myoblasts with ILK siRNA also antagonized the potentiating effect of TNF-α (Fig. 2A,B,E). In addition, transfection of cells with ILK siRNA reduced ILK protein expression (Fig. 2D). We thus directly measured the ILK kinase activity in response to TNF-α stimulation by the immunoprecipitation of ILK from lysates. Figure 2F shows that TNF-α exposure in myoblasts time dependently increased ILK kinase activity, assessing the phosphorylation of the recombinant GSK3β on Ser9. Therefore, ILK plays an important role in TNF-α-induced iNOS expression.

THE SIGNALING PATHWAYS OF Akt AND mTOR ARE INVOLVED IN THE POTENTIATING ACTION OF TNF-α

It has been reported that ILK is an upstream regulator of the phosphorylation of Akt on Ser473 [Troussard et al., 2003], we then examined whether TNF-α stimulation also enhances the association of ILK with Akt. Stimulation of myoblasts with TNF-α increased Akt phosphorylation time and dose dependently (Fig. 3A,B). Pretreatment of cells for 30 min with Akt inhibitor (1L-6-hydroxymethyl-

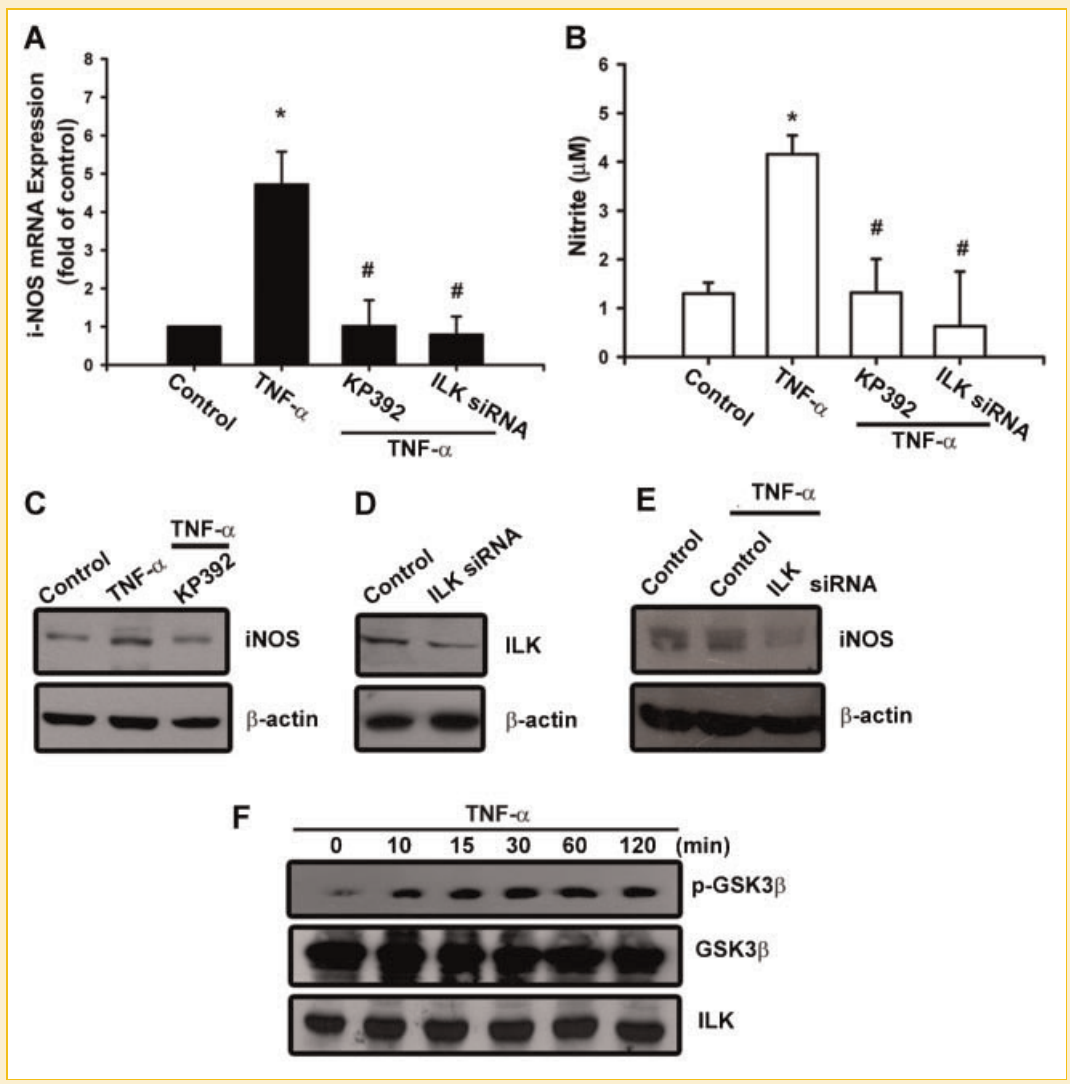

Fig. 2. ILK activation is involved in TNF-α-induced iNOS expression. A,B: Cells were pretreated with ILK inhibitor of KP-392 (10 μM) for 30 min or transfected with ILK siRNA for 24 h followed by stimulation with TNF-α (10 ng/ml) for 24 h, the mRNA level of iNOS and NO production were determined by qPCR and Griess reaction. C: Cells were pretreated with ILK inhibitor of KP-392 (10 μM) for 30 min followed by stimulation with TNF-α (10 ng/ml) for 24 h, the protein level of iNOS was determined by Western blot. D: Cells were transfected with control or ILK siRNA for 24 h, the protein expression of ILK was examined by Western blot analysis. E: Cells were transfected with ILK siRNA for 24 h followed by stimulation with TNF-α (10 ng/ml) for 24 h, the protein level of iNOS was determined by Western blot. F: Cells were exposed to TNF-α (10 ng/ml) for indicated time intervals, and cell lysates were immunoprecipitated (IP) with an antibody specific for ILK. Immunoprecipitated proteins were separated by SDS–PAGE and immunoblotted with anti-pGSK3β or GSK3β. Results are expressed of four independent experiments. $^{*}P < 0.05$ as compared with control. $^{\#}P < 0.05$ as compared with TNF-α-treated group.

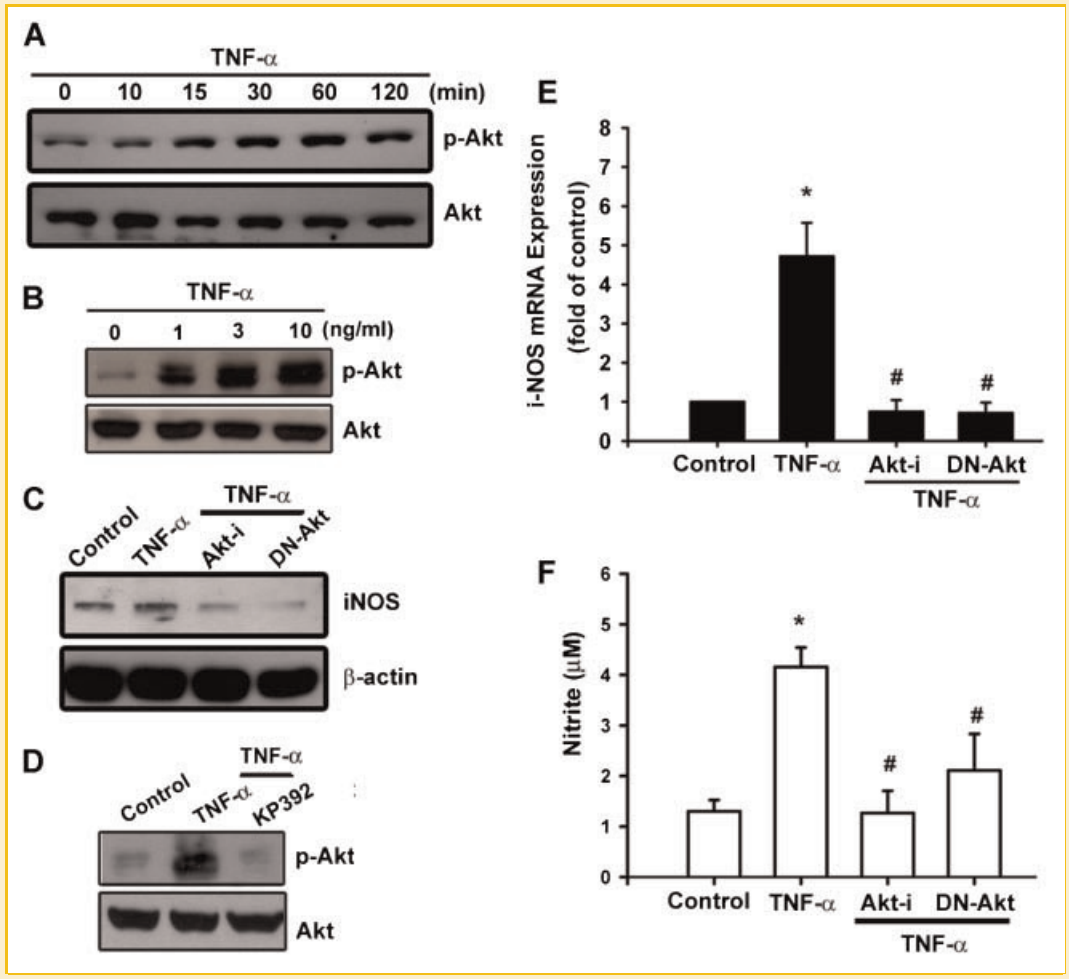

Fig. 3. Akt is involved in TNF-α-mediated iNOS expression in myoblasts. Cells were incubated with TNF-α (10 ng/ml) for various time intervals (A) or incubated with TNF-α for different concentration for 120 min (B), cell lysates were then immunoblotting with an antibody specific for phosphor-Akt. Cells were pretreated for 30 min with Akt inhibitor (10 μM) or transfected with Akt mutant or vector for 24 h followed by stimulation with TNF-α for 24 h, the protein and mRNA expression of iNOS and NO production were examined by Western blot (C), qPCR (E), and Griess reaction (F). D: Cells were pretreated with KP392 for 30 min followed by stimulation with TNF-α for 15 min, the p-Akt expression was examined by Western blot. Results are expressed of four independent experiments. $^{*}P < 0.05$ as compared with control. $^{\#}P < 0.05$ as compared with TNF-α-treated group.

chiro-inositol-2-[(R)-2-*O*-methyl-3-Ooctadecylcarbonate] inhibited the TNF-α-induced iNOS expression and NO production (Fig. 3C,E,F). In addition, treatment of cells with Akt inhibitor (10 μM) did not affect cell viability, which was assessed by the MTT assay (Supplementary Fig. S2). Transfection of cells with Akt mutant also reduced TNF-α-mediated iNOS expression and NO production (Fig. 3C,E,F). On the other hand, TNF-α-induced Akt phosphorylation markedly decreased if myoblasts were pretreated for 30 min with KP-392 (Fig. 3D). Therefore, Akt may function as a downstream signaling molecule of ILK in the TNF-α signaling pathway. To examine whether mTOR activation is involved in the elevation of iNOS expression caused by TNF-α stimulation, the mTOR inhibitor rapamycin was used. Pretreatment of cells with rapamycin antagonized the TNF-α-induced increase of iNOS expression and NO production (Fig. 4A–C). Transfection of cells with mTOR siRNA also reduced TNF-α-mediated iNOS expression and NO production (Fig. 4A–C). Transfection of cells with mTOR siRNA reduced mTOR expression (Fig. 4D). After exposure to TNF-α the mTOR phosphorylation on Ser2448 increased at 10 min (Fig. 4E). On the other hand, TNF-α-induced mTOR phosphorylation markedly decreased if myoblasts were pretreated for 30 min with KP-392 and Akt inhibitor (Fig. 4F). These effects implicate the involvement of ILK/Akt-dependent mTOR activation in TNF-α-mediated induction of iNOS. Taken together, these results indicate that the Akt and mTOR pathways are involved in TNF-α-induced iNOS expression.

INVOLVEMENT OF NF-κB IN TNF-α-INDUCED iNOS EXPRESSION

NF-κB activation has been reported to be necessary for iNOS induction in macrophages [Monsalve et al., 2009]. To examine whether NF-κB activation is involved in the signal transduction pathway caused by TNF-α that leads to iNOS expression, the NF-κB inhibitor PDTC was used. Figure 5A,B shows that PDTC (30 μM) inhibited the enhancement of iNOS expression and NO production

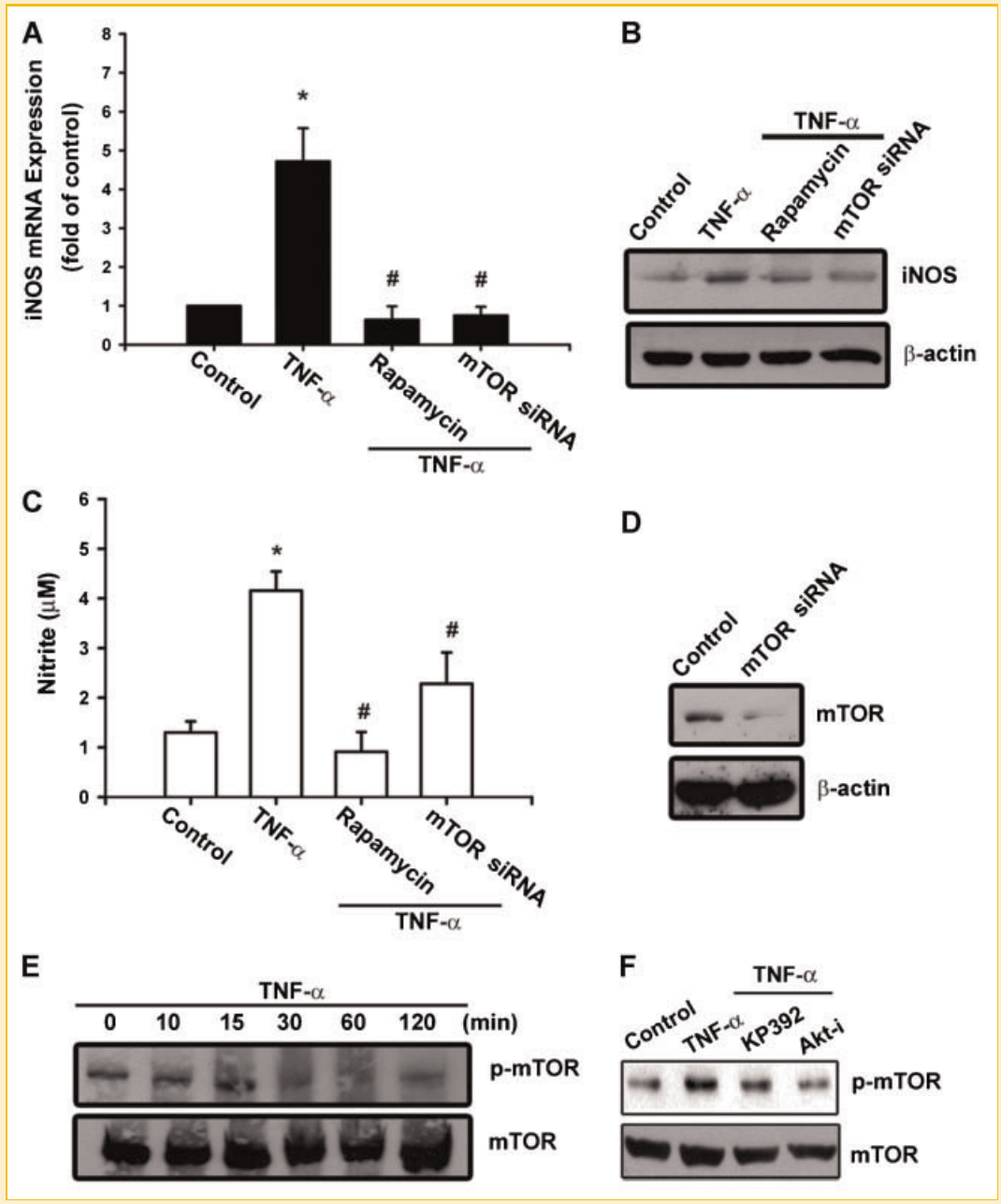

Fig. 4. mTOR is involved in TNF-α-mediated iNOS expression in myoblasts. Cells were pretreated for 30 min with rapamycin (100 nM) or transfected with mTOR siRNA for 24 h followed by stimulation with TNF-α for 24 h, the mRNA and protein expression of iNOS and NO production were examined by qPCR (A), Western blot (B), and Griess reaction (C). D: Cells were transfected with control or mTOR siRNA for 24 h, the protein expression of mTOR was examined by Western blot analysis. E: Cells were incubated with TNF-α (10 ng/ml) for various time intervals, cell lysates were then immunoblotting with an antibody specific for phosphor-mTOR. F: Cells were pretreated with KP392 or Akt-i for 30 min followed by stimulation with TNF-α for 15 min, the p-mTOR expression was examined by Western blot. Results are expressed of four independent experiments. $^{*}P < 0.05$ as compared with control. $^{\#}P < 0.05$ as compared with TNF-α-treated group.

induced by TNF-α. Furthermore, pretreatment of cells with an IκB protease inhibitor [L-1-tosylamido-2-phenylenylethyl chloromethyl ketone (TPCK, 3 μM)] antagonized the potentiating action of iNOS and NO (Fig. 5A,B). In addition, treatment of cells with PDTC (30 μM) or TPCK (3 μM) did not affect cell viability, which was assessed by the MTT assay (Supplementary Fig. S2). We further examined the upstream molecules involved in TNF-α-induced NF-κB activation. Stimulation of cells with TNF-α-induced IKKα/β phosphorylation in a time-dependent manner (Fig. 5C). Furthermore, transfection with IKKα or IKKβ mutant markedly inhibited TNF-α-induced NO production (Fig. 5D). These data suggest that IKKα/β activation is involved in TNF-α-induced NO production in murine myoblasts. Treating myoblasts with TNF-α also caused IκBα phosphorylation in a time-dependent manner (Fig. 5C). Previous studies showed that p65 Ser^{536} phosphorylation increases NF-κB transactivation [Madrid et al., 2001], and the antibody specific against phosphory-

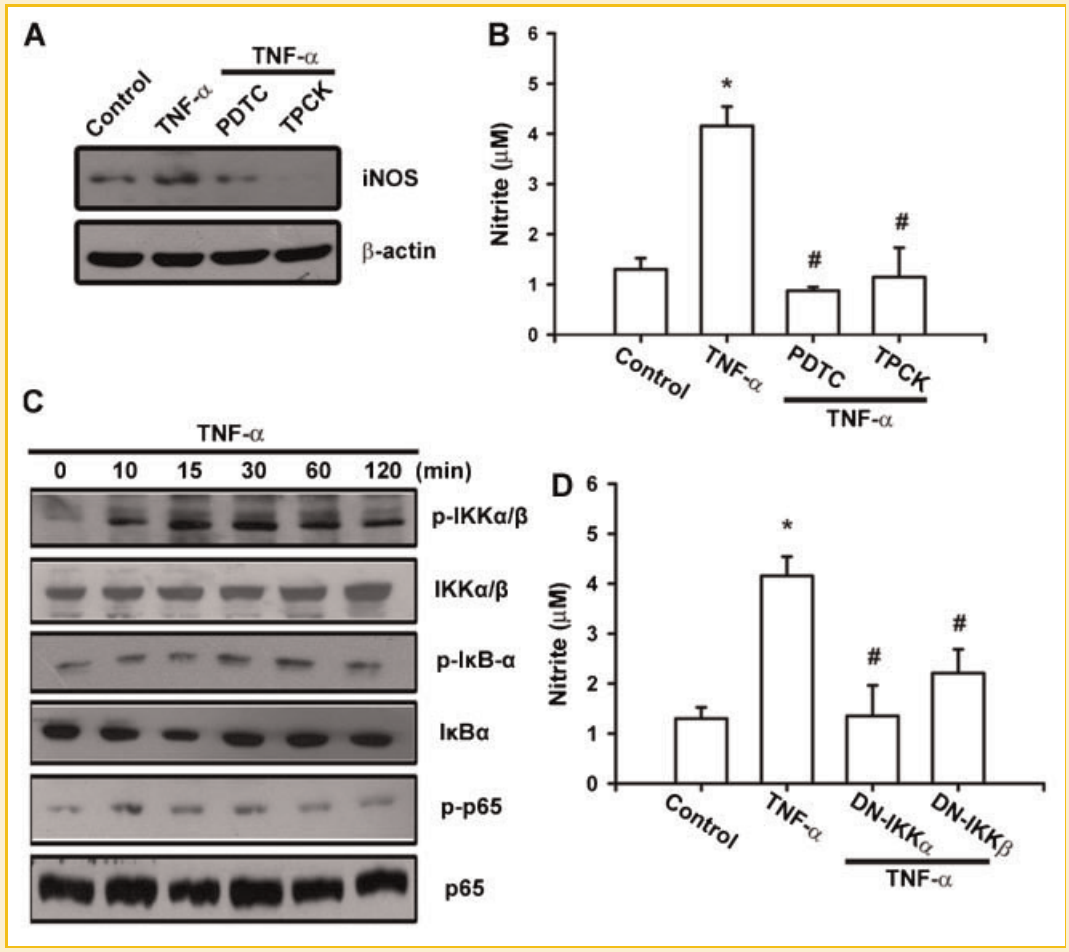

Fig. 5. TNF-α induces iNOS expression through NF-κB. Cells were pretreated for 30 min with PDTC (10 μM) or TPCK (3 μM) followed by stimulation with TNF-α, the protein expression of iNOS and NO production were examined by Western blot (A) and Griess reaction (B). Cells were incubated with TNF-α for indicated time intervals, and p-IKK, p-IκBα, and p-p65 expression was determined by Western blot analysis (C). Cells were transfected with IKKα and IKKβ mutant for 24 h before incubation with TNF-α for 24 h. The NO production was examined by Griess reaction (D). Results are expressed of four independent experiments. $^{*}P < 0.05$ as compared with control. $^{\#}P < 0.05$ as compared with TNF-α-treated group.

lated p65 Ser^{536} was employed to examine p65 phosphorylation. Treatment of cells with TNF-α for various time intervals resulted in p65 Ser^{536} phosphorylation (Fig. 5C).

ILK, Akt, AND mTOR PATHWAYS ARE MEDIATED TNF-α-INDUCED NF-κB ACTIVATION

It has been reported that the NF-κB binding site is important for the activation of the iNOS gene [Hou et al., 2009]. NF-κB activation was further evaluated by analyzing the electrophoretic mobility shift assay. Stimulation of cells with TNF-α resulted activation of NF-κB-specific DNA–protein complex formation (Fig. 6A). Pretreatment of cells with KP392, Akt inhibitor, or rapamycin reduced TNF-α-mediated NF-κB-specific DNA–protein complex formation (Fig. 6A). To identify the specific subunit involved in the formation of the NF-κB complex, supershift assay was performed using antibody specific for anti-p65. Incubation of nuclear extracts with anti-p65 antibody attenuated NF-κB DNA–protein binding (Supplementary Fig. S3). These results indicated the component of p65 in NF-κB complex. To further investigate whether TNF-α-induced NF-κB activation occur through the ILK, Akt, and mTOR pathways, G7 cells were pretreated for 30 min with KP392, Akt inhibitor and rapamycin, which inhibited the TNF-α-induced increase in κB-luciferase activity (Fig. 6B). In addition, co-transfection of cells with ILK siRNA, Akt mutant, and mTOR siRNA also reduced TNF-α-mediated κB-luciferase activity (Fig. 6C). Stimulation of cells with TNF-α increased p65 translocation into nucleus by immunofluorescence staining (Fig. 7A). KP392, Akt inhibitor, and rapamycin reduced TNF-α-mediated translocation of p65 (Fig. 7A).

To further study the pathways involved in the action of TNF-α-induced iNOS expression, transient transfection was performed using the mouse iNOS promoter-luciferase construct, piNOS-Luc, which contains the mouse iNOS gene between positions −1592 and +171 fused to the luciferase reporter gene. Exposure to TNF-α led to a 2.8-fold increase in iNOS promoter activity in myoblasts. The increase of iNOS activity by TNF-α stimulation was antagonized by KP382, Akt inhibitor, rapamycin, PDTC, and TPCK (Fig. 7B). In co-transfection experiments, the increase of iNOS promoter activity by TNF-α was inhibited by the ILK siRNA, Akt mutant, mTOR siRNA,

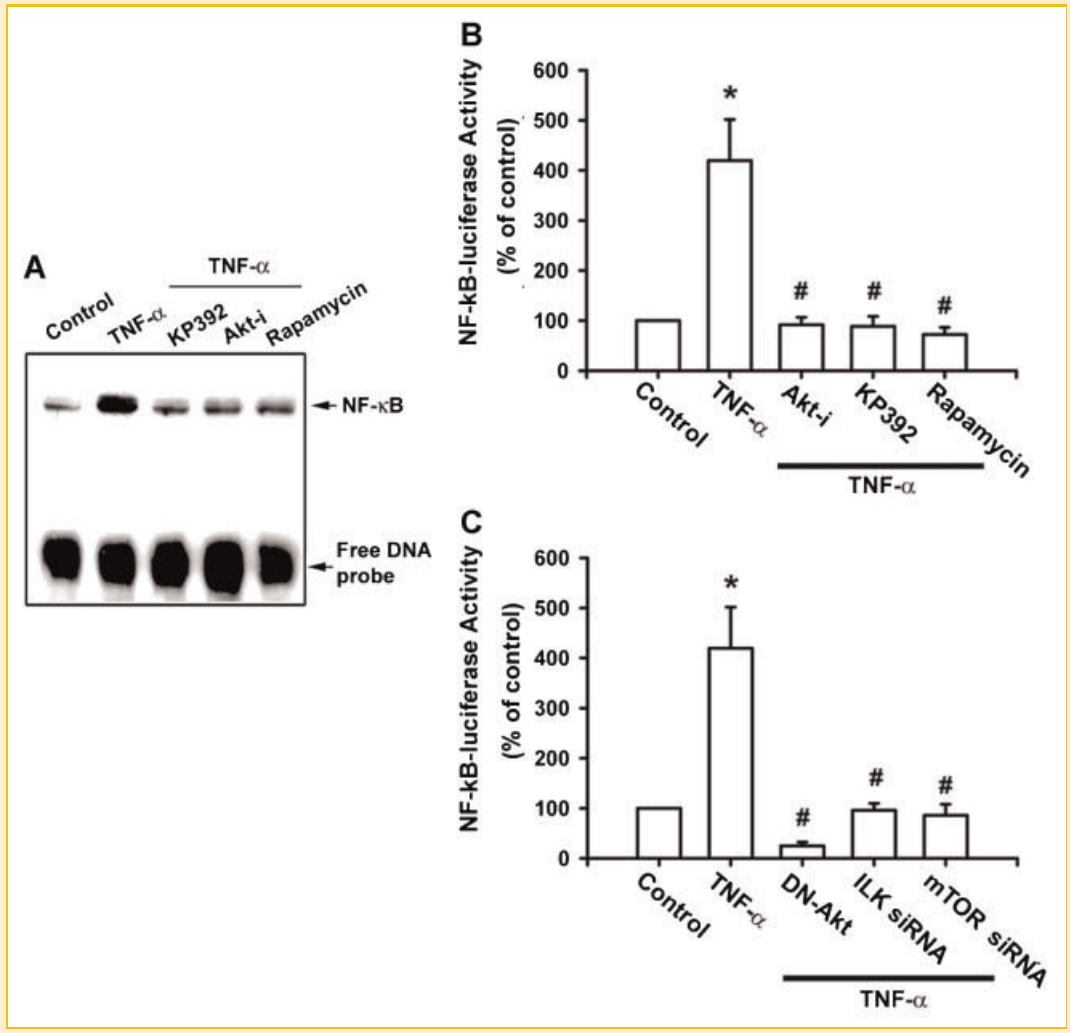

Fig. 6. TNF-α induces NF-κB activation in myoblasts. A: Cells were pretreated for 30 min with KP392, Akt inhibitor, or rapamycin followed by stimulation with TNF-α for 120 min, and electrophoretic mobility shift assay was performed as described in the Materials and Methods Section. Cells were pretreated with KP392, Akt inhibitor, and rapamycin for 30 min before exposure to TNF-α (B), cells were transfected with ILK siRNA, Akt mutant, and mTOR siRNA before exposure to TNF-α (C). The NF-κB-luciferase activity was measured, and the results were normalized to the β-galactosidase activity and expressed as the mean ± SE for three independent experiments performed in triplicate. $^{*}P < 0.05$ as compared with control. $^{\#}P < 0.05$ as compared with TNF-α-treated group.

IKKα mutant, and IKKβ mutant (Fig. 7C). Taken together, these data suggest that the activation of the ILK/Akt/mTOR/NF-κB pathway is required for the TNF-α-induced increase of iNOS in myoblasts.

DISCUSSION

TNF-α is a pleiotropic cytokine produced by activated macrophages and other cells [Balkwill, 2009; Bastos et al., 2009]. TNF-α can induces necrosis, metabolic alteration, and large spectrum of cell function disfunction including changes in anaerobic metabolism [Jurisic et al., 2005]. Previous studies on the effects of the cytokine TNF-α on muscle cells rely on the hypothesis that loss of skeletal muscle, associated with chronic or inflammatory disorders may result from an inability of satellite cells to differentiate into functional fibers after damage or degeneration. It has been reported that TNF-α induces the expression of inflammatory cytokines through activation of the transcription factor NF-κB [Huang et al., 2003; Balkwill, 2009]. However, the signaling pathway for TNF-α in iNOS expression and NO production in myoblasts is mostly unknown. Here we further identified iNOS as a target protein for the TNF-α signaling pathway that regulates the cell inflammatory response. We also showed that potentiation of iNOS by TNF-α requires activation of the ILK, Akt, mTOR, and NF-κB signaling pathway. These findings suggest that TNF-α acts as an inducer of inflammatory cytokines such as iNOS and enhance the inflammatory response. To further examine these effects are mediated by TNF system, the TNF-α antibody (Ab) was used. Pretreatment of cells with TNF-α Ab reduced TNF-α-mediated nitrite production, NF-κB activity, ILK activity, Akt, and mTOR phosphorylation (Supplementary Fig. S1). Therefore, these effects are mediated by TNF-α system.

ILK has been reported to regulate LPS and ultrasound-mediated iNOS expression [Tan et al., 2002; Tang et al., 2007]. The current study showed that TNF-α stimulation increased kinase activity of

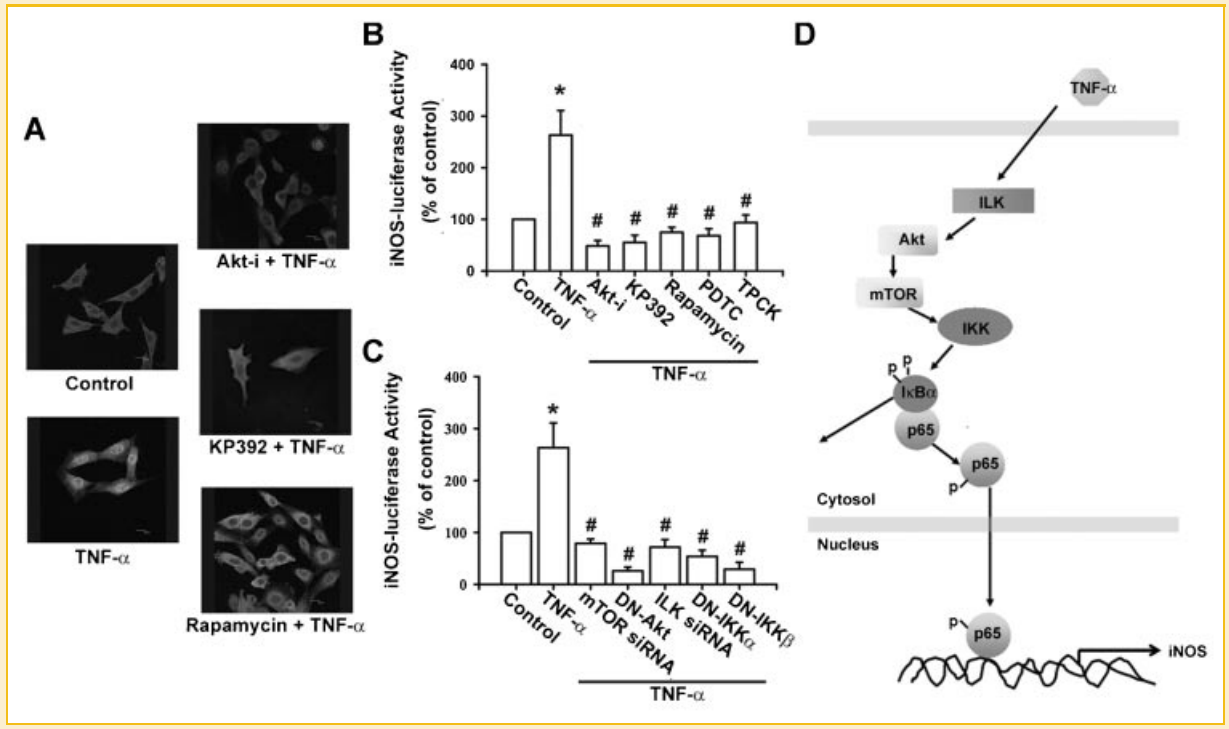

Fig. 7. ILK/Akt and mTOR pathways are involved in TNF-α-mediated NF-κB activation and iNOS expression. A: Cells were pretreated with KP392, Akt inhibitor, and rapamycin for 30 min, they were followed by stimulation with TNF-α for 120 min, and p65 immunofluorescence staining was examined. Cells were pretreated with KP392, Akt inhibitor, rapamycin, PDTC, and TPCK for 30 min before exposure to TNF-α (B), cells were transfected with ILK siRNA, Akt mutant, mTOR siRNA, IKKα mutant, and IKKβ mutant before exposure to TNF-α (C). The iNOS-luciferase activity was measured, and the results were normalized to the β-galactosidase activity and expressed as the mean ± SE for three independent experiments performed in triplicate. $^{*}P < 0.05$ as compared with control. $^{\#}P < 0.05$ as compared with TNF-α-treated group. D: Schematic diagram of the signaling pathways involved in TNF-α-induced NO production in myoblasts. TNF-α increases iNOS expression by activation of ILK, Akt, mTOR which enhances binding of p65 to the NF-κB site, resulting in the transactivation of iNOS expression and NO production.

ILK. Treatment with the ILK inhibitor of KP-392 inhibited TNF-α-induced iNOS expression and NO production. Furthermore, the ILK siRNA also antagonized the TNF-α-mediated potentiation of iNOS expression. Therefore, ILK activation is involved in TNF-α-induced iNOS expression in the cultured myoblasts. ILK possibly regulated the cell function by promoting the phosphorylation of Akt on Ser473 and its downstream pathways of mTOR [Persad et al., 2001]. Our results demonstrate that pretreatment of myoblasts with Akt or mTOR inhibitors antagonized the increase of iNOS expression and NO production under TNF-α stimulation. This was further confirmed by the result that the dominant negative mutant of Akt and mTOR siRNA inhibited the enhancement of iNOS promoter activity under TNF-α stimulation. Here we also found that the cytoplasmic kinase Akt and mTOR were activated by TNF-α stimulation in myoblasts. These effects implicate the involvement of ILK, Akt, and mTOR activation in TNF-α-mediated induction of iNOS and NO. Activation of the ILK/Akt/mTOR-dependent pathway has also been reported to regulate vascular epidermal growth factor in tumor angiogenesis [Tan et al., 2004]. In addition, regulation of tumor growth by ILK inhibitor (QLT0254) is also related to the ILK/Akt/mTOR signal cascade [Yau et al., 2005]. Taken together, our results provide evidence that TNF-α up-regulates iNOS in the cultured myoblasts via the ILK/Akt/mTOR signaling pathway.

A variety of growth factors stimulate the expression of iNOS via signal-transduction pathways that converge to activate NF-κB complex of transcription factors [Monsalve et al., 2009]. The results of this study show that NF-κB activation contributes to TNF-α-induced iNOS expression and NO production in myoblasts, and that the inhibitors of the NF-κB-dependent signaling pathway, including PDTC and TPCK inhibit TNF-α-induced iNOS expression. Upon stimulation, such as by TNF-α, IκB proteins become phosphorylated by the multisubunit IKK complex, which subsequently targets IκB for ubiquitination, and are then degraded by the 26S proteasome. Finally, the free NF-κB translocates to the nucleus, where it activates the responsive gene [Baldwin, 1996]. In the present study, we found that treatment of myoblasts with TNF-α resulted in increases in IKKα/β phosphorylation, and the binding of p65 to NF-κB element. Using transient transfection with κB-luciferase as an indicator of NF-κB activity, we also found that TNF-α-induced an increase in NF-κB activity. The IKKs can be stimulated by various proinflammatory stimuli, including IL-1β and peptidoglycan [Hatada et al., 2000]. These extracellular signals activate the IKK complex, which is comprised of catalytic subunits (IKKα and IKKβ) and a linker subunit (IKKγ/NEMO). This kinase complex phosphrylates IκBα at Ser^{32} and Ser^{36} and signals for ubiquitin-related degradation [Chen et al., 1995]. The released NF-κB is then translocated into the nucleus where it promotes NF-κB-dependent transcription [Baldwin, 1996]. There also is strong evidence that IKKα and IKKβ are themselves phosphorylated and activated by one or more upstream activating kinases [Hatada et al., 2000]. p65 is

phosphorylated at Ser^{536} by a variety of kinases through various signaling pathways, and this enhances the p65 transactivation potential. TNF-α induces rapid p65 phosphorylation at Ser^{536} through IKKs, resulting in increased transcriptional activity of p65 [Sakurai et al., 1999]. The results of this study showed that TNF-α increased the phosphorylation of IKKα/β, IκBα, and p65. On the other hand, KP392, Akt inhibitor, and rapamycin reduced TNF-α-mediated p65 translocation into nucleus, NF-κB-specific DNA–protein complex formation, and NF-κB promoter activity. Our data indicated that ILK/Akt/mTOR and NF-κB pathways might play important role in the expression of iNOS of murine myoblasts.

In conclusion, we explored the signaling pathway involved in TNF-α-induced iNOS expression and NO production in myoblasts. We found that TNF-α increases iNOS expression by activating of ILK, Akt, and mTOR which enhances binding of p65 to the NF-κB site and results in the transactivation of iNOS expression and NO production (Fig. 7D).

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