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TGF-β SUPPRESSES THE EXPRESSION OF GENES RELATED TO MITOCHONDRIAL FUNCTION IN LUNG A549 CELLS

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Abstract

TGF-β is a mediator of lung fibrosis and regulates the alveolar epithelial type II cell phenotype. TGF-β can induce epithelial mesenchymal transition of idiopathic pulmonary disease and cancer metastasis. Peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1 α) is a key metabolic regulator that stimulates mitochondrial biogenesis and promotes remodeling of muscle tissue to oxidative fiber-type composition. Here, we report that the induction of TGF-β decreased mRNA expression of PGC-1α, and PGC-1 target genes, such as the transcription factors NRF-2, ERR-α, and PPAR-γ in lung epithelial A549 cells. In addition, TGF-β led to the reduction of super oxide dismutase 2 (anti-oxidant enzyme), cytochrome C (electron transport chain in mitochondria), and MCAD (a mitochondrial β-oxidant enzyme) in A549 cells. Together, our results suggest that TGF-β may suppress the transcriptional activity of the genes related to mitochondrial biogenesis or function. This mechanism may provide a novel insight into the understanding of fibrosis disease.

Key words:TGF-β; PGC-1α; mitochondrial biogenesis.

Article information

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INTRODUCTION

TGF-β is a pleiotropic growth factor that plays an important role in cell proliferation, cell growth arrest, senescence, apoptosis, and differentiation (21, 36). TGF-β plays dual roles in cancer: first, acting as a tumor suppressor by preventing tumorigenesis in breast and prostate cancer (2), and second, enhancing the invasiveness and metastasis of tumor cells(17). TGF-β also mediates epithelial-to-mesenchymal transition (EMT), which is a mediator for tissue fibrosis and tumor progression. EMT mediated by TGF-β which alters the epithelial properties results in fibrosis in the kidney, liver, or lung (10).

TGF-β is an important factor in the pathogenesis of fibrosis. Asbestosis is a form of pulmonary fibrosis, which results from the entry of asbestos into the lung via inhalation. Asbestos-induced fibrogenesis results in the production of reactive oxygen species (ROS) related to the biological activity of TGF-β in A549 cells (20). Idiopathic pulmonary fibrosis (IPF) occurs in the usual interstitial pneumonia resulting from enhanced TGF-β signaling(12). Asbestosis and IPF share similarities with respect to histopathological appearance and radiographic manifestations (4). IPF occurs as a result of alveolar epithelial injury and fibroblast-myofibroblast foci, and abnormal wound healing (26). TGF-β signaling activates nuclear translocation of phosphorylation of Smad2/Smad3. TGF-β signaling also affects apoptosis and gene expression resulting from the activation of p38 and c-Jun N-terminal kinases (JNK) and mitogen-activated protein kinases (34, 37).

Mitochondria dysfunction is related to the onset and progression of many neurodegenerative, cardiovascular, and metabolic disorders, as well as the development of certain types of cancer (5, 9, 14, 31). The peroxisome proliferator-activated receptor (PPAR) co-activator (PGC-α) regulates mitochondrial function, biogenesis and adaptation (13, 28, 29). Activation of mitochondrial biogenesis by PGC-1α is modulated by the co-activation of ERR-α (estrogen-related receptor alpha), NRF-1(nuclear respiratory factor), and NRF-2 (16). Replication and expression for mtDNA are regulated by NRF-1, NRF2, SP1, YY1 (yin yang protein 1), and ERR-α, which promote the expression of genes encoding mitochondrial proteins (22).

Our study showed that induction of TGF-β cause the downregulation of mRNA expression of PGC-1, and its related genes. The results of our study suggest that TGF-β may act as a repressor for the transcriptional activity of genes related to the mitochondrial enzymes in lung A549 cells.

MATERIALS AND METHODS

Cell culture

A549 cells (donated by Dr. Moon, Pusan National University) were incubated in RPMI (Roswell Park Memorial Institute) medium (Invitrogen, Gibco Cell Culture, Portland, OR, USA) supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Prior to stimulation with TGF-β, the cells were washed with phosphate buffered saline (PBS), and then treated with 2 ng/mL of TGF-β in serum-free RPMI media containing penicillin and streptomycin.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted using Iso-RNA Lysis reagent (5Prime.Inc, Gaithersburg, MD,USA) according to the manufacturer's protocol. cDNA was generated by iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules,

CA,USA). qRT-PCR analysis was performed using the ICycler device (Bio-Rad Laboratories) with SYBR Green supermix (Bio-Rad Laboratories) according to the manufacturer's instructions with the following primers following as PGC-1 forward, GTCACCACCCAAATCCTTAT; PGC-1 backward, ATCTACTGCCTGGAGACCTT, PPAR-γ forward, GGCTTCATGACAAGGGAGTTTC; PPAR-γ backward, AACTCAAACTTGGGCTCCATAAAG; NRF-1 forward, CCACGTTACAGGGAGGTGAG; NRF-1 backward, TGTAGCTCCCTGCTGCATCT; NRF-2 forward, TACTCCCAGGTTGCCCACA; NRF-2 backward, CATCTACAAACGGGAATGTCTGC, ERRα forward, CCTCTGTGACCTCTTTGACC; backward: TACTGACATCTGGTCAGAC; SOD1 forward, GGTCCTCACTTTAATCCTCTAT; SOD1 backward, CATCTTTGTCAGCAGTCACATT; SOD2 forward, TGACAAGTTTAAGGAGAAGC; SOD2 backward, GAATAAGGCCTGTTGTTCC, Actin forward, TGAAGTGTGACGTGGACATC; Actin backward; GGAGGAGCAATGATCTTGAT. Data analysis was completed using the gene expression analysis program from iCycler iQ Real-Time PCR. All mRNA data are given as a ratio normalized with GAPDH.

Western blotting

The cells were washed with ice-cold PBS and introduced into the sample buffer. Total cell lysates were separated with 7.5% bis-tris gel and transferred onto the polyvinylidene fluoride membrane (Bio-Rad Laboratories). The membrane was incubated with primary antibodies PGC-1α (Santa Cruz Biotechnology, CA, USA), ERR-α (Santa Cruz Biotechnology), Actin (Cell Signaling Technologies, Beverly, MA, USA), and subsequently detected using HRP-labeled IgG conjugates. HRP-conjugated secondary antibodies were detected with a chemiluminescence detection system.

Immunocytochemistry and imaging

A549 cells stimulated TGF-β (2 ng/mL) or media for 24hr were washed twice with PBS and fixed with 3.7 % formaldehyde for 10 min at room temperature. After washing twice with PBS, the cells were permeabilized with chilled 90% methanol for 5 min. The cells were blocked by 2% bovine serum albumin (BSA) in PBS for 30 min. PGC-1α (Santa Cruz Biotechnology) or ERR-α antibody (Santa Cruz Biotechnology) was incubated overnight in 2% BSA. The cells were washed 3 times with PBS and incubated for 30 min with Alex Fluor 568 goat rabbit-IgG antibody (Invitrogen) (1:500 dilution). Nuclei were stained with DAPI (1:1000). Images were captured with a Laser confocal microscope (Olympus).

RESULTS

A549 has been previously used to study the mechanism of tumorigenesis and apoptosis in lung cancer cells (1, 3, 8). A recent study revealed that A549 lung cancer cells led to EMT transition by TGF-β (11, 19). In the present study, we first investigated the morphological changes to A549 cells following treatment with 2 ng/mL TGF-β. The A549 cells were cultured for 24 h in the presence of TGF-β, and their morphology was studied using phase-contrast microscopy. We observed that the A549 cells showed fibroblast-like, spindle-shaped morphology in the treatment of TGF-β (Figure 1A) , but the expression of vimentin or E-cadherin, which are both markers for the EMT was not observed. These results are consistent with a previous study(36) showing that TGF-β caused the EMT phenotype in A549 cells, but did not affect either the vimentin or E-cadherin.

It has been suggested that TGF-β activity suppresses the ROS generated from the mitochondria, but the role of mitochondrial biogenesis or function by induced TGF-β in the lung cells has not been investigated. To determine whether TGF-β plays a role in mitochondrial biogenesis, we first determined the mRNA expression of PGC-1α. PGC-1α is a key modulator of mitochondrial biogenesis. Our analysis of the mRNA levels of PGC-1α by RT-PCR indicated that TGF-β treatment in A549 cells downregulated the mRNA expression of PGC-1α (Figure 1B). This downregulation of PGC-1α was verified by comparing with a control medium using qRT-PCR (Figure 1C). In addition, western blotting revealed that PGC-1α reduced during the treatment of lung A549 cells with TGF-β (Figure 2B), suggesting that TGF- β may regulate the transcription of PGC-1α.

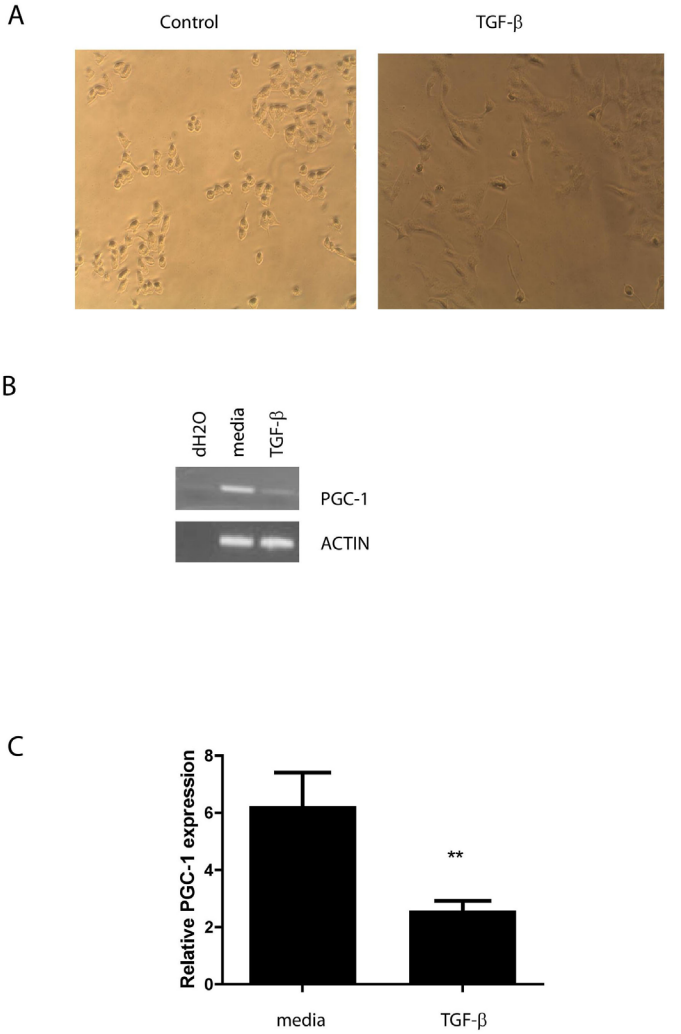

Figure 1. TGF-β suppressed the transcriptional activity of PGC-1α in A549 cells. A) TGF-β induced the morphological change in A549 cells. Two nanograms of TGF- β was added to the A549 cells and incubated for 24 hr. Left panel shows the medium control, and right panel shows the cells treated with TGF-β. B) RT-PCR analysis of PGC-1α mRNA expression during the 24-h-long TGF-β treated A549 cells. Actin serves as the loading control. C) qRT-PCR analysis of PGC-1 mRNA expression following 24-h treatment of A549 cells with TGF-β. N = 4. Error bars show the mean ± SER. $^{**}P < 0.01$

PGC-1α as a transcriptional co-activator functions in the regulation of cellular energy metabolism. We examined the effect of TGF-β on the transcriptional genes of PGC-1α. PGC-1α serves as a transcriptional activator for NRF-1, NRF-2, PPAR-γ, and ERR-α. Using qRT-PCR, we determined the mRNA expression of NRF-1, NRF-2, PPAR-γ, and ERR-α in the treatment of A549 cells with TGF-β. Our results showed that induction of TGF-β in A549 cells suppressed mRNA expression of NRF-2 and PPAR-γ, but that of NRF-1 did not change (Figure 2 A).

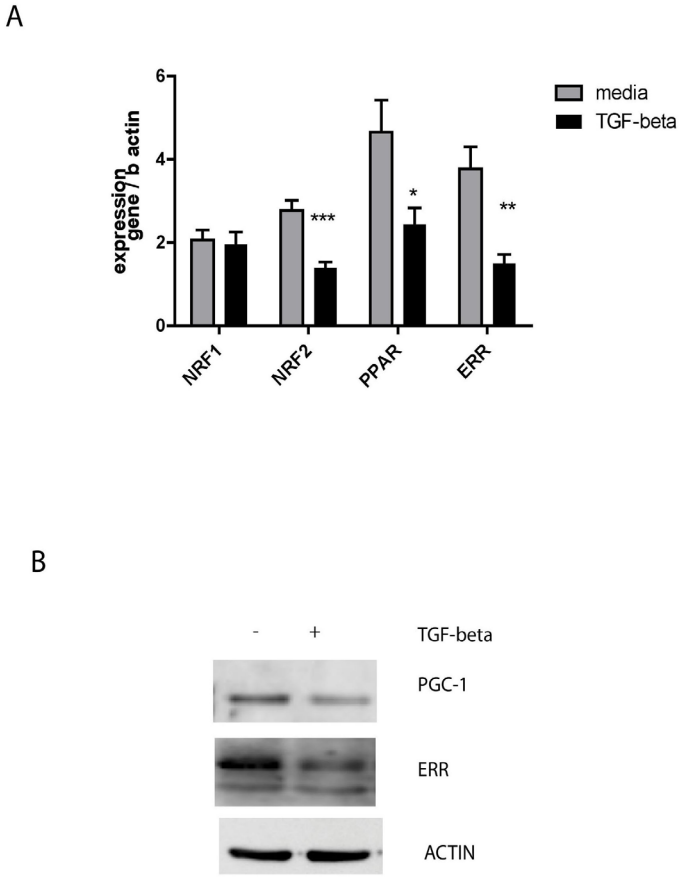

Figure 2. TGF-β suppressed the expression of target genes of PGC-1α. A) qRT-PCR analysis for the target genes of PGC-1α. A549 cells were incubated with TGF-β (2 ng/mL) or medium for 24 h, and the total RNA were analyzed. The mRNA expression level for the transcriptional target genes of PGC-1α was determined by qRT-PCR analysis. Error bars show the mean ± SER. N = 4. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$. B) Protein expression of PGC-1α and ERR-α. A549 cells were incubated with TGF-β (2 ng/mL) or medium for only 24 h, and the lysate was immunoblotted with antibodies against PGC-1α, ERR-α, and actin antibodies.

The ERR family is the main PGC-1α partner which plays a role in the regulation of the mitochondrial and tissue-specific oxidative metabolic pathways (7, 23, 25, 35). By immunoblotting, we observed that ERR-α was decreased following TGF-β treatment of A549 cells (Figure 2B). Following immunostaining, we examined the images of ERR-α by using confocal microscopy, and observed decreased expression of ERR-α following treatment with TGF-β compared to the medium control cells (Figure 3A). We further examined the subcellular localization of ERR-α by using high magnification (1200×) of confocal microscopy and determined that ERR-α was mainly localized in the cytoplasm. Interestingly, we observed that ERR-α was localized in both the nucleus and cytoplasm in some A549 cells following TGF-β treatment (Figure 3B, lower panel), suggesting that TGF-β may be responsible for the shuttling of ERR-α from the cytoplasm into the nucleus.

We also examined the effect of TGF-β on the genes related to the mitochondrial antioxidant enzymes, and several target genes of PPAR, ERR-α, and NRF-2. Both PPAR-γ and ERR-α regulate MCAD (medium-chain acyl-CoA dehydrogenase), which catalyzes the initial step in mitochondrial fatty acid β-oxidation (27).

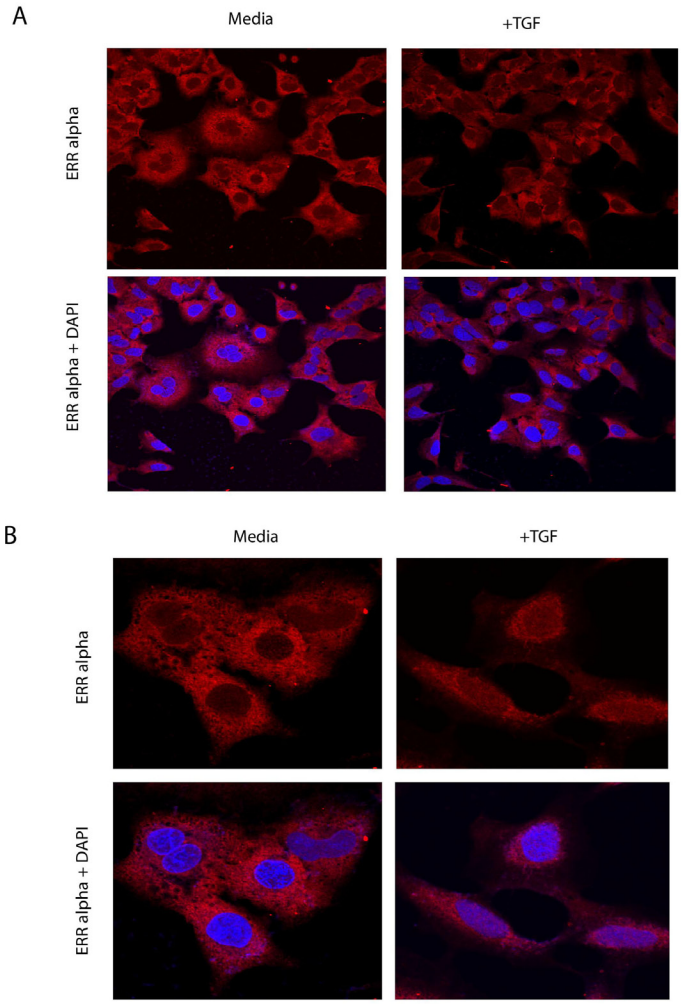

Figure 3. TGF-β causes the shuttling of ERR-α from the cytoplasm into the nucleus. Cellular localization of ERR-α in A549 cells. A549 cells were incubated with TGF-β (2 ng/mL) or medium only for 24 h, and the cells were immunostained with antibodies against with ERR-α. Images were observed by confocal microscopy. A) Confocal microscopy images showing downregulation of ERR-α in the presence of TGF-β in A549 cells (magnification, 400×). B) Confocal microscopy images (magnification, 1200×) showing nuclear localization of ERR-α for the treatment of A549 cells with TGF-β.

NRF-2 activates transcription of the COX subunit II (mitochondrion-encoded) and COX subunit IV (nucleus-encoded), which function in the process of generating energy (6). The copper/zinc SOD (Cu/ZnSOD or SOD1) is a cytosolic enzyme, and manganese superoxide dismutase (MnSOD or SOD2) is a mitochondrial antioxidant enzyme. Cytochromc C is released from mitochondria during the early stages of apoptosis. Therefore, we determined the expression levels of MCAD, CytoC, COXII, COXIV, SOD1, and SOD2 by qRT-PCR. Our data revealed that the expression of SOD2, MCAD, and cytochrome C was reduced following TGF-β treatment of lung A549

cells, but that of SOD1, COXII, COXIV did not change (Figure 4). Our results imply that TGF-β may regulate the genes related to mitochondrial function, biogenesis or mitochondrial antioxidant enzymes.

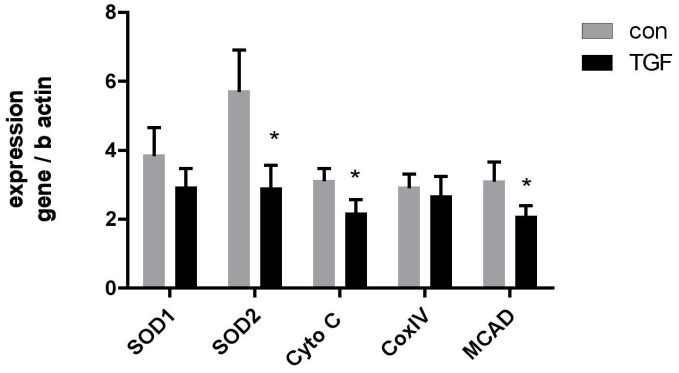

Figure 4. TGF-β suppressed the genes for mitochondrial antioxidant enzymes and metabolism enzymes. qRT-PCR showing the decrease of mRNA expression of SOD-2, CytoC, and MCAD. A549 cells were incubated with TGF-β (2 ng/mL) or medium for only 24 h. The mRNA levels of SOD-1, SOD2, CytoC, COXII, COX IV, and MCAD were determined by qRT-PCR. N = 4. Error bars show the mean ± SER. $*P < 0.05$.

DISCUSSION

The role of TGF-β signaling has been extensively studied in cancer, apoptosis, and fibrosis. TGF-β has been found to increase the activity of ROS in lung cells (15), but its role in mitochondrial biogenesis, which is the main source of ROS, was not previously known. Our results indicate that TGF-β suppresses the transcriptional activity of the mitochondrial genes in lung A549 cells.

Reportedly, TGF-β causes mitochondrial defects in the Mv1Lu lung epithelial cells (36). Our data imply that TGF-β plays a role as the repressor for the transcriptional regulator of PGC-1α or mitochondria related genes and may regulate mitochondrial biogenesis by downregulating PGC-1α, NRF-2, PPAR-γ, and ERR-α in lung A549 cells. PGC-1α has been known as a major transcriptional regulator of the mitochondrial detoxification system, and it also affects the heart, liver, fat, and brain by moderating the mitochondrial genes (24). PGC-1α regulates mitochondrial biogenesis through the nuclear respiratory factors (NRFs) and co-activates the transcriptional function of NRF-1 (32). ERR-α, as a partner of PGC-1α, functions in regulating cellular energy balance by targeting MCAD, which mediates mitochondrial beta-oxidation of fatty acyl acids (27, 30). Our findings indicate that TGF-β has the ability to cause ERR-α shuttling from the cytoplasm into the nucleus. ERR family is similar to Estrogene receptors (ER). Further studies are required to better understand the mechanism of shuttling of ERR-α by TGF-β.

Recent studies have reported that the Smad family, which is a mediator of TGF-β signaling acts as a repressor of PGC-1α expression, and the white adipose tissue from smad3 deficient mice showed an increase in mitochondrial biogenesis (33). There is evidence that Smad4 has high interaction affinity with COXII from yeast two-hybrid screening following TGF-β or UV stimulation (18). Our findings imply the possibility that TGF-β suppresses the transcriptional activity for the mitochondrial antioxidant enzymes and metabolic enzymes such as SOD2, CytoC, and MCAD. However, further investigation will be necessary to determine whether the transcriptional activity of mitochondrial gene can be regulated by the Smad family.

In summary, our results showed that TGF-β, which is known as key factor of EMT in fibrosis suppresses the mRNA expression of PGC-1α and the genes related to mitochondrial biogenesis. This study provides a novel insight into the role of TGF-β as a repressor for mitochondrial biogenesis in fibrosis.

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