HIF-1α Modulate Transcriptional Regulation of Telomerase Reverse Transcriptase (TERT) in the Pulmonary Arterial Smooth Muscle Cells*

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Abstract: Objective To explore the role of hypoxia-inducible factor-1α(HIF-1α) to the expression of telomerase reverse transcriptase (TERT) in the pulmonary arterial smooth muscle cells under anoxia. **Methods** HIF-1α and TERT mRNA in the pulmonary arterial smooth muscle cells were examined by RT-PCR with the presence of HIF-1α inducers, inhibitor or oligodeoxynucleotides. HIF-1α protein was examined by Western blot analysis. **Results** Exposure of PASMCs to hypoxia produced a remarkable increase in HIF-1α and TERT mRNA levels. HIF-1α inducers $CoCl_2$ and DFX markedly increased mRNA expression of HIF-1α, and hypoxia-induced upregulation of HIF-1α and TERT was inhibited by FAS, HIF-1α inhibitor. CBZ-LLL significantly increased TERT mRNA expression, but it had no effect on the mRNA levels of HIF-1α. The decoy of HIF-1α by its specific binding dsODN blocked the $CoCl_2$-induced increase in TERT mRNA levels in these PASMCs. **Conclusion** TERT expression in PASMCs is transcriptionally regulated via an HIF-1α pathway under hypoxic conditions.

Key words: hypoxia-induciblefactor-1α; genetranscription; anoxia; telomerase reverse transcriptase

INTRODUCTION

Hypoxia is an important regulator of physiologic processes, including erythropoiesis, angiogenesis and glycolysis[1]. In the vasculature, chronic hypoxia has been shown to cause proliferation of vascular smooth muscle cells(VSMC), leading to vessel wall remodeling, a key pathophysiologic component of pulmonary hypertension[2]. It was previously reported that VSMC express high levels of telomerase activity when exposed to hypoxia and demonstrate the role of telomerase activation in long-term growth of VSMC under chronic hypoxia[3]. The enzyme telomerase, comprising a telomerase reverse transcriptase (TERT) subunit and a RNA subunit (TR), is ubiquitous from unicellular protozoa to mammals[4]. Transcriptional regulation of TERT is the major mechanism of telomerase activation[5]. While telomerase activity is an important factor in cell proliferation, hypoxia exposure has been shown to increase TERT gene expression, suggesting that telomerase activation may also be a mechanism that protects against genetic stress induced by hypoxia[6]. However, the molecular mechanisms by which hypoxia activates telomerase have not been examined in any detail.

Hypoxia-inducible factor-1 (HIF-1) is a helix-loop-helix transcriptional factor, which consists of HIF-1α and HIF-1β, has been cloned and characterized as a transcriptional activator of many oxygensensitive genes, such as erythropoietin, vascular endothelial growth factor, transferrin, and several glycolytic enzymes[7]. It has been

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indicated that HIF-1α is an inducible protein by a decrease in tissue or cellular O_2. HIF-1β is not inducible, but it can be bound to HIF-1α to form a dimer to activate the transcription of many genes containing *cis* hypoxia-response element (HRE) in their promoter or enhancer regions. Interestingly, computer-assisted homology searches have revealed potential binding sites for HIF-1α in the TERT proximal promoter.

Based on these observations, we propose that hypoxia may induce proliferation of pulmonary arterial smooth muscle cells(PASMC), mediated by TERT through HIF-1α.The present study was designed to test if HIF-1α is involved in hypoxia-induced activation of the TERT promoter in the rat PASMC. We provide direct evidence that induction of TERT promoter activity by hypoxia is mediated by HIF-1α through HRE in the TERT proximal promoter. This study adds TERT to the list of hypoxia-inducible genes regulated by this transcription factor.

MATERIALS AND METHODS

1. Culture of PASMCs

Pulmonary arterial smooth muscle cells (PASMCs) of rat were cultured by the method of Chamley-Campbell et al[8]. Male Sprague-Dawley rats weighing between 100 g and 110 g (n=8) were anesthetized with pentobarbital sodium (30 mg/kg, administered ip). The heart and lung were extracted using a sterile technique and put in a culture dish with D-Hanks' fluid (Hanks' solution free of calcium and magnesium anion). The extrapulmonary artery was isolated, and the endothelium was removed. After two washes with D-Hanks' fluid, the middle lamella smooth muscle was cut into 1 mm^3 blocks, which were cultured using the method of Chamley-Campbell[8]. PASMCs reached confluence in 4-6 days in medium RPMI-1640 with 20% fetal calf serum (FCS). A standard staining method as described previously[9] was used to confirm the identity of these cells. PASMC of passages 3 and 6 were plated in 100 mm^2 tissue culture dishes in 5 ml of media 24 h before the experiments to form a subconfluence. To decrease PO_2 in the culture medium, the dishes were transferred to a sealed, humidified modular chamber and flushed for 2 h or 4 h with 5% CO_2-95% N_2. PO_2 in the culture medium was measured by the method of Morita[10]. After hypoxia, the culture medium was rapidly replaced by TRIzol solution, and then total RNA was extracted.

2. RNA Extraction and Real-time Reverse Transcription(RT)-PCR Analysis

Total cellular RNA was isolated by using Isogen reagent (Nippon Gene, Tokyo, Japan) and reverse transcribed into cDNAs by using a First-strand cDNA Synthesis kit (Roche Applied Science). Real-time PCR was performed for quantitative estimation of TERT mRNA and HIF-1α. The primers for HIF-1α were sense 5'-ACTATGTCGCTTTCTTGG -3'; and antisense 5'- GTTTCTGCTGCCTTGTAT -3'. (accession No. NM024359). the resultant amplicon was 195bp long. The primers for TERT were sense 5'-TGTTCCTGTTCTGGCTAATG-3'; and antisense 5'-TGCTTGACCTCCTCTTGTG-3'. (accession No. AY539717), and the resultant amplicon was 196 bp long. Real-time-PCR was performed in a total volume of 10 μL per reaction. We placed 1 μL of cDNA into a 9 μL reaction mixture that contained 0.1 μL of Taq DNA polymerase (5 U/μL, Platinum DNA Polymerase; Invitrogen), 1 μL of the supplied 10x PCR buffer, 0.5 μL of $MgCl_2$ (50 mmol/L), 0.2 μL of dNTPs (10 mmol/L; Fermentas), 0.15 μL of bovine serum albumin (10 g/L; Serva), 0.5 μL of the appropriate sense primer, 0.5 μL of the corresponding antisense primer (3 μmol/L), and 1 μL of the TaqMan probe (3 μmol/L); finally, DEPC-H_2O was added to a final volume of 10 μL. The cycling protocol consisted of an initial 5-min denaturation step at 88°C, followed by 50 cycles of denaturation at 94°C for 10 s, annealing at 55°C for 20 s, and extension at 72°C for 20 s. The PCR products were subjected to electrophoresis in 2% agarose gels, stained with ethidium bromide, and

visualized under UV light. The expression of β-actin mRNA was amplified in parallel as control for RNA loading and RT-PCR efficiency.

3. Western Blot Analysis

Cells were harvested and lysed on ice for 30 min in lysis buffer (10 mM Tris[pH 8.0], 1 mM EDTA, 400 mM NaCl, 10% glycerol, 0.5% NP-40, 5 mM sodium fluoride, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol) containing complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, Ind.). Equal amounts of protein (40 g) were loaded onto a sodium dodecyl sulfate-polyacrylamide gel (4 to 12% polyacryl amide) and subjected to electrophoresis at 200 V for 50 min. The protein was transferred onto a polyvinylidene difluoride membrane and probed with anti-TERT antibodies (H-231; rabbit IgG; Santa Cruz Biotechnology), anti-HIF-1α antibody (H-206; rabbit IgG; Santa Cruz Biotechnology), and anti-actin antibody (H-196; rabbit IgG; Santa Cruz Biotechnology). The same blot was probed after the membrane was stripped with the different antibodies. Each protein was detected by horseradish peroxidase-conjugated secondary antibody coupled with enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, Piscataway, N. J.).

4. Decoy of HIF-1

It has been demonstrated that HIF-1α activates gene expression by binding to a promoter or an enhancer site, HRE. This cis element contains a -CGTG- consensus sequence. A standard fluorescein-attached HRE containing oligodeoxynucleotides(ODN) was synthesized with sequences of 5’- GACCAGCGTGCATAGCTA -3’(sense) and 5’-TAGCTATGCACGCTGGTC-3’ (antisense) and scrambled ODN with sequences of 5’- GACCAGGCAGCCATAGCTA -3’ (sense) and 5’- TAGCTATGGCTGCCTGGTC -3’ (antisense)[11]. The fluorescein attachment at the 5’-end was used as an indicator for transfection into the cells. To make double-strand ODN (dsODN), both sense and antisense ODNs (100 μM in TE, pH 8.0)were heated at 95°C for 5 min and then cooled slowly down to room temperature[12]. These dsODNs were wrapped by using cationic liposomes (GenePORTER Transfection Reagent; GTS) and transfected into PASMCs as described by the manufacturer. dsDNA (10 μg) was first mixed with 50 μl of liposome and then added to 5 ml of serum-free incubation medium. The transfection efficiency was evaluated by a fluorescence microscope (Olympus, Tokyo, Japan) 24 h after incubation of PASMCs with the liposome-dsODN mixtures. Positively transfected cells (60%–80% cells), indicated by a remarkable intracellular fluorescence, were used to determine the effects of the HIF-1α decoy on the gene expression of TERT induced by hypoxia or HIF-1α inducers and to prepare the nuclei for in vitro transcription and nuclear protein extraction.

5. Statistical Analysis

Data are presented as means SE. The significance of the difference in mean values within and among multiple groups was examined with an ANOVA for repeated measures followed by a Duncan’s post hoc test. Student’s t-test was used to evaluate the significance of differences between two groups of experiments (SigmaStat, SPSS). A value of $P<0.05$ was considered statistically significant.

RESULTS

1. Effect of Hypoxia on TERT and HIF-1α mRNA Expression in PASMCs

Fig. 1A presents the results of RT-PCR analysis of HIF-1α and TERT mRNA. Exposure of PASMCs to hypoxia produced a remarkable increase in HIF-1α and TERT mRNA levels. Fig. 1B summarizes the data from these experiments. Densitometric analysis shows that reduction of PO_2 in the culture medium for 2 h snd 4h

significantly increased mRNA levels of HIF-1α (n=6) and TERT (n=6) in PASMCs.

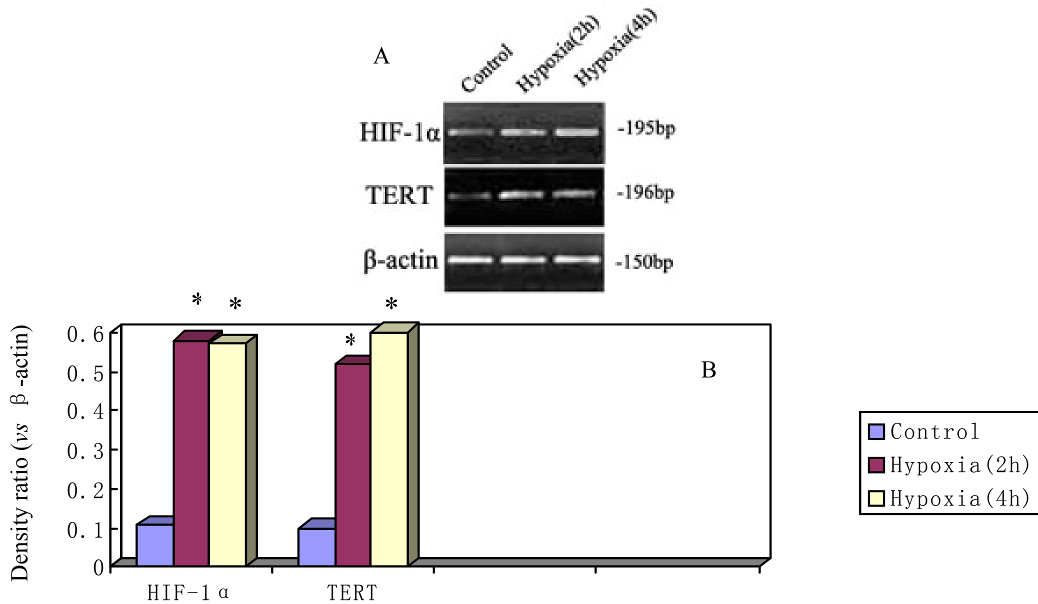

Fig. 1 Effects of hypoxia on telomerase reverse transcriptase (TERT) and hypoxia-inducible factor-1α (HIF-1α) mRNA expression in pulmonary arterial smooth muscle cells (PASMCs)

Notes: A. HIF-1α and TERT mRNA expression in PASMCs with and without hypoxic treatment by RT-PCR; B. Summarized data showing the intensity of TERT and HIF-1α blots; *$P<0.05$ compared with control.

2. Effect of HIF-1α Induction on TERT mRNA Expression in PASMCs

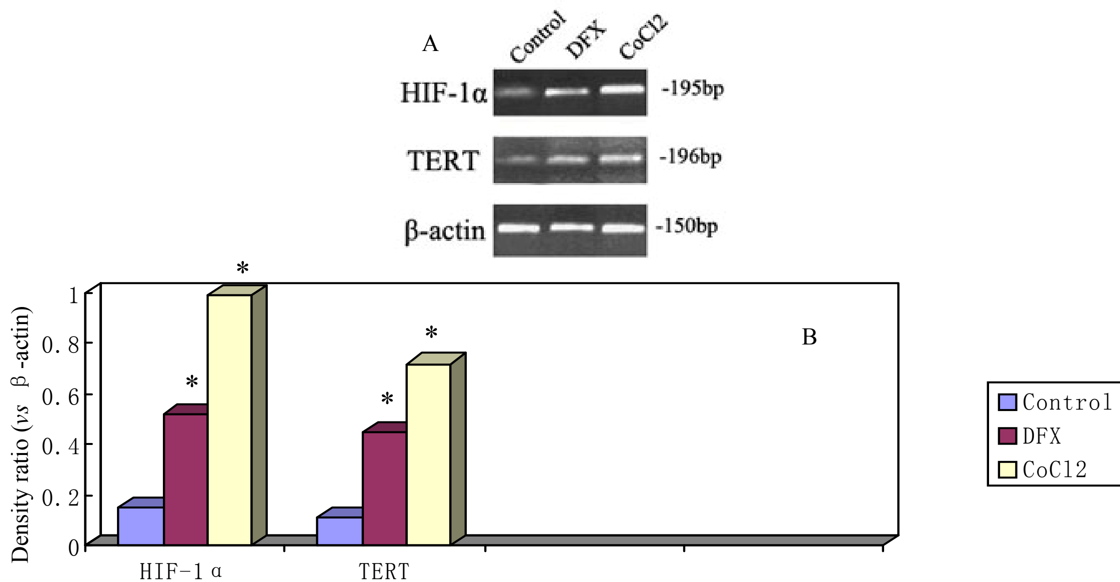

Fig. 2 Effects of HIF-1α induction by $CoCl_2$ and DFX on TERT and HIF-1α mRNA expression in PASMCs

Notes: A. HIF-1α and TERT mRNA expression in PASMCs with and without $CoCl_2$ and DFX by RT-PCR; B. Summarized data showing intensity of TERT and HIF-1α blots. *$P<0.05$ compared with control.

We performed the experiments to observe whether HIF-1α inducers Cobalt chloride($CoCl_2$) and desferrioxamine (DFX) increase the levels of TERT mRNA in PASMCs. These cells were incubated with 150 μM $CoCl_2$ and 260 μM DFX for 4 h, and then total RNA was extracted. Fig. 2A shows a typical gel document of

HIF-1α and TERT expression by RT-PCR. A significant increase in HIF-1α mRNA was found in $CoCl_2$-or DFX -treated PASMCs. In parallel, TERT mRNA levels were increased in these cells. These results are summarized in Fig.2B.HIF-1α inducers $CoCl_2$ and DFX markedly increased mRNA expression of HIF-1α (n=6) and TERT (n=6).

3. Effect of HIF-1α Expression Inhibition on TERT mRNA Levels in PASMCs

It has been reported that ferrous ammonium sulfate(FAS) is an inhibitor of HIF-1α expression, which blocked DFX-mediated induction of HIF-1α[13]. The present study determined the effect of FAS on HIF-1α expression and TERT mRNA levels during cell hypoxia. In these experiments, PASMCs were incubated in a hypoxic chamber for 4 h in the presence or absence of 200 µM of FAS. As shown in Fig. 3A, PASMCs subjected to hypoxia more abundantly expressed HIF-1α and TERT compared with control cells. In the presence of FAS, both HIF-1α and TERT mRNAs were not altered in response to cell hypoxia. These results are summarized in Fig. 3B. Hypoxia-induced upregulation of HIF-1α (n=6) and TERT(n=6) was inhibited by FAS.

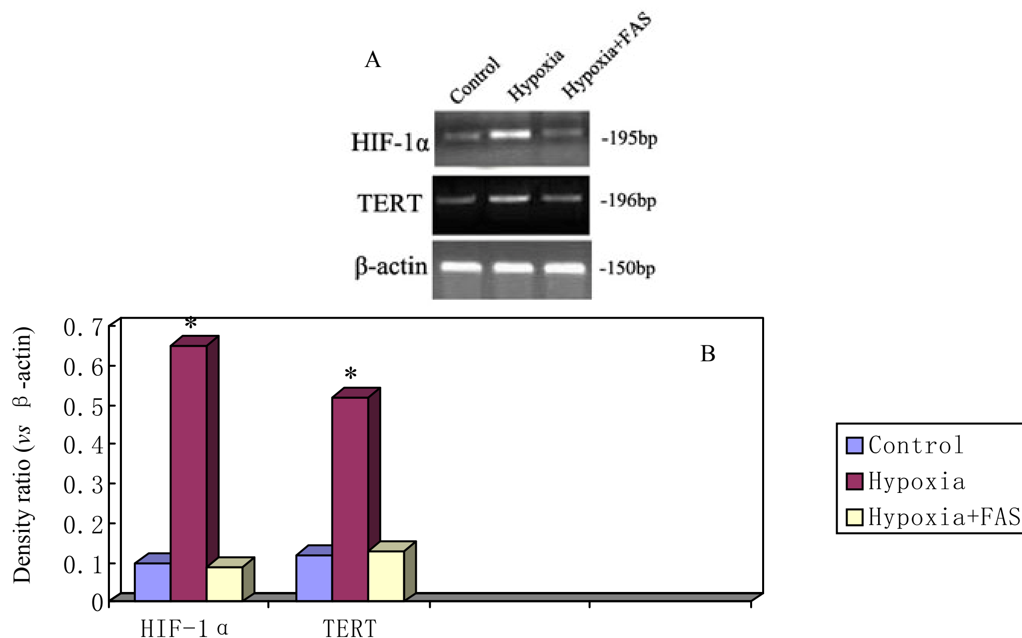

Fig. 3 Effects of HIF-1α inhibition by ferrousammoniumsulfate (FAS) on TERT an HIF-1α mRNA expression in PASMCs

Notes: A. HIF-1α and TERT mRNA expression in PASMCs with and without FAS by RT-PCR;
B. Summarized data showing intensity of TERT and HIF-1α blots; *$P<0.05$ compared with control.

4. Effect of Ubiquitin-proteasome Inhibition on TERT mRNA Expression in PASMCs

Recent studies have indicated that HIF-1α is degraded via the ubiquitin-proteasome pathway[14]. Therefore, alteration of HIF-1α protein levels by blocking the aforementioned protease pathway may change TERT mRNA expression. In the present study, *N*-carbobenzoxyl-L-leucinyl-L- leucinyl-L-norvalinal (CBZ-LLL) was used to specifically inhibit ubiquitin-proteasome, and then the effect on TERT mRNA expression was observed. Pretreatment of PASMCs with 10 µM CBZ-LLL for 4 h dramatically increased TERT mRNA levels in these cells, even under normoxic conditions. Because CBZ-LLL primarily acted to block the degradation of HIF-1α protein, HIF-1α mRNA levels were not altered by this protease inhibition. The above results are summarized in Fig. 4B. CBZ-LLL significantly increased TERT mRNA expression (n=6), but it had no effect on the mRNA levels of HIF-1α .

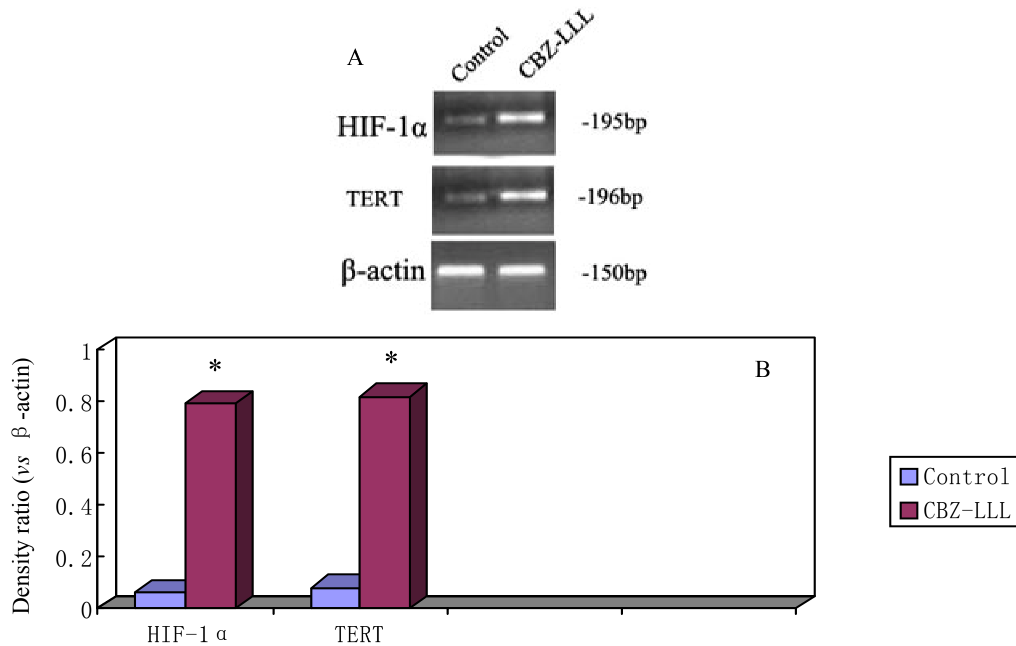

Fig. 4 Effects of HIF-1α degradation inhibitor CBZ-LLL on TERT and HIF-1α MRNA expression in PASMCs
Notes: A. HIF-1α and TERT mRNA expression in PASMCs with and without CBZ-LLL by RT-PCR;
B. Summarized data showing intensity of TERT and HIF-1α blots; **P*<0.05 compared with control.

5. Effect of Hypoxia, $CoCl_2$, and CBZ-LLL on HIF-1α Protein Expression in PASMCs

By Western blot analysis, HIF-1α protein expression was also found to increase significantly in PASMCs exposed to hypoxia for 4 h, suggesting that increased transcripts during hypoxia are used to produce HIF-1α protein. Similarly, the treatment of PASMCs with 150 μM $CoCl_2$ for 4 h produced a significant increase in HIF-1α protein levels in these cells. Moreover, inhibition of proteasome by 10 μM CBZ-LLL markedly increased HIF-1α levels in PASMCs. All of these results are summarized in Fig. 5.

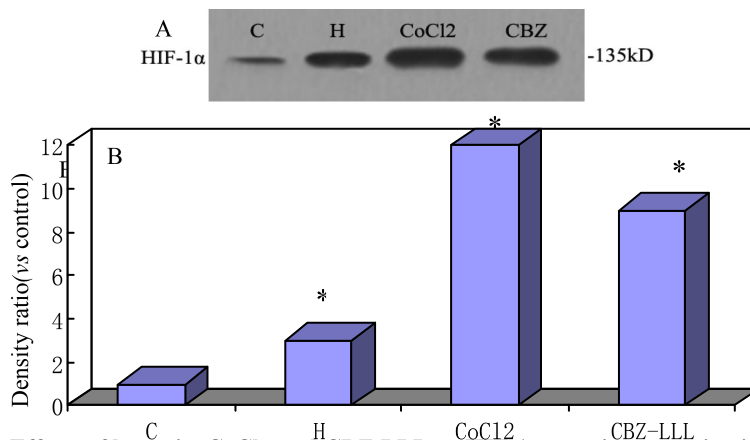

Fig. 5 Effects of hypoxia, $CoCl_2$, and CBZ-LLL on HIF-1α protein expression in PASMCs
Notes: A. HIF-1α protein expression in PASMCs exposed tohypoxia for 4 h or in the presence of 150 μM $CoCl_2$ and 10 μm CBZ-LLL for 4 h. A band at 135kD is identified to be HIF-1α; B. Summarized data showing the intensity of immunoreactive band of HIF-1α protein;**P*<0.05 compared with control.

6. Effect of HIF-1α Decoy on TERT mRNA Expression in PASMCs

As shown in Fig. 6, 60%-80% of the PASMCs were transfected by fluorescein dsODN (Fig. 6). These cells were used to determine the effects of dsODNs on TERT mRNA expression in response to hypoxia or $CoCl_2$. As

shown in Fig. 8A, hypoxia induced TERT mRNA expression. However, the hypoxia-induced increase in TERT mRNA levels was depressed in the cells transfected with specific dsODN [dsODN(+)] containing 5'-CGTG-3'. As a control, scrambled dsODN [dsODN(-)] without 5'-CGTG-3' was used to transfect PASMCs, but it had no effect on the increase in TERT mRNA levels induced by hypoxia (Fig. 7B). Fig. 8C summarizes the results of these experiments. Clearly, the decoy of HIF-1α by its specific binding dsODN blocked the induction of TERT mRNA. Similarly, the decoy of HIF-1α was found to block the $CoCl_2$-induced increase in TERT mRNA levels in these PASMCs. As shown in Fig. 8A, $CoCl_2$ increased TERT mRNA levels. In dsODN(+)-transfected cells, the increase of TERT mRNA induced by $CoCl_2$ was markedly reduced. Fig. 8B summarizes the results of these experiments. The decoy of HIF-1α, by introduction of its binding sequence, produced a remarkable blockade of $CoCl_2$-induced TERT upregulation.

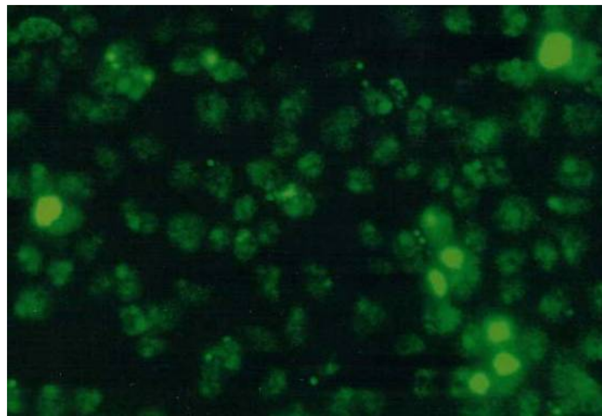

Fig. 6 Fluorescent photomicrograph of PASMCs transfected with fluorescein-attached dsODN containing HIF-1α binding site under fluorescence microscope (×200)

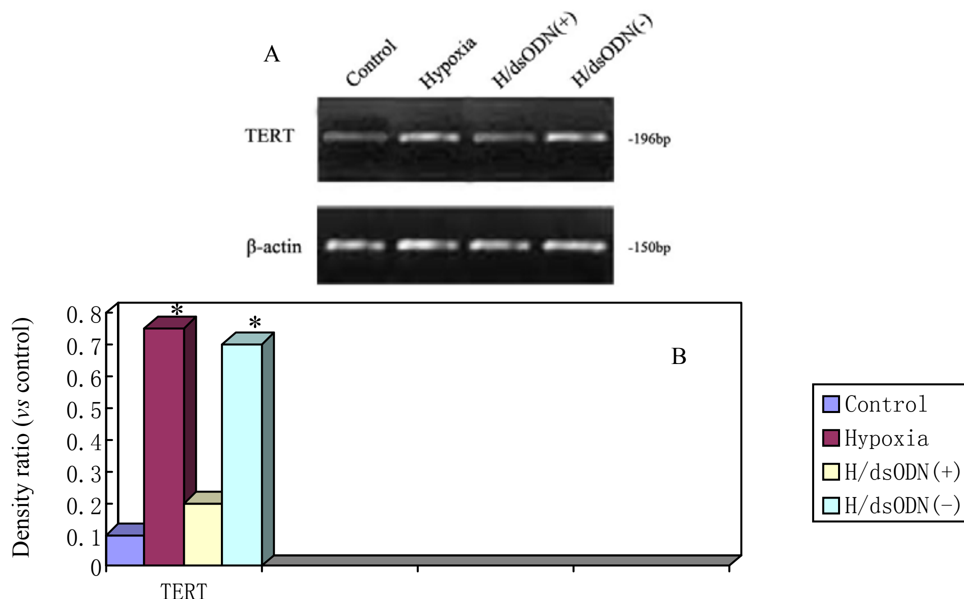

Fig. 7 Effects of HIF-1α decoy by introduction of synthetic dsODN on TERT mRNA expression in response to hypoxia in PASMCs

Notes: A. RT-PCR analysis using synthetic dsODN for TERT mRNA. dsODN(+),5'-CGTG-3' containing dsODN. dsODN(-), Scrambled dsODN without 5'-CGTG-3'; B. Summarized data showing intensity of TERT blots; **P*<0.05 compared with control; $^{\triangle}$*P*<0.05 compared with values obtained from hypoxic cells.

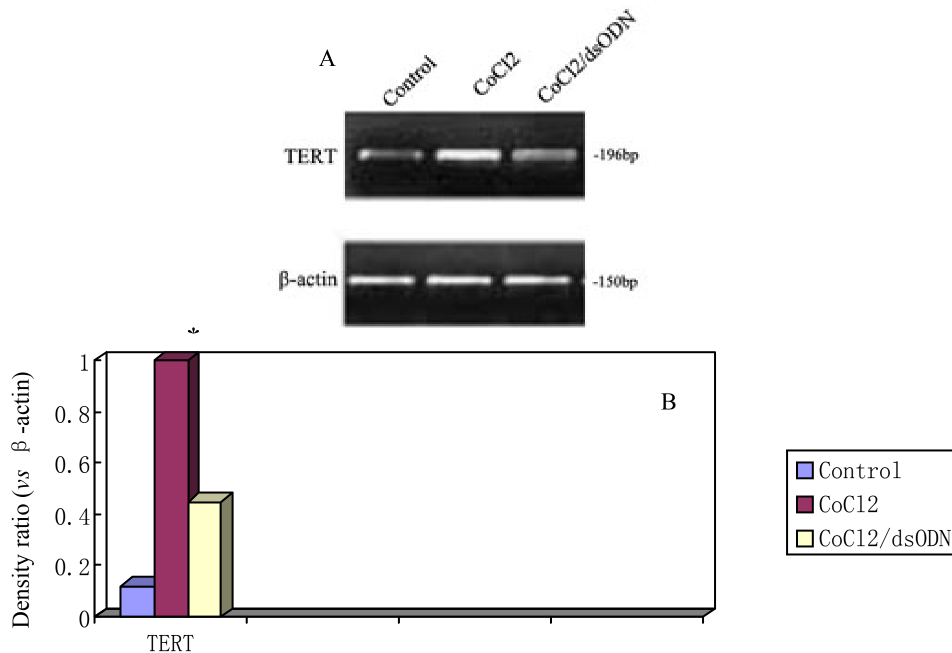

Fig. 8 Effects of HIF-1α decoy by introduction of synthetic dsODN on TERT mRNA expression in the presence of $CoCl_2$ in PASMCs

Notes :A. RT-PCR analysis for TERT mRNA in the presence of $CoCl_2$; B. Summarized data showing intensity of TERT blots; **P*<0.05 compared with control; △*P*<0.05 compared with values obtained from $CoCl_2$ –treated cells.

DISCUSSION

In the present study, TERT mRNAs were detected in cultured PASMCs. When these cells were exposed to hypoxia for 2 or 4 h, TERT mRNA levels was markedly increased. These results suggest that TERT is a hypoxia-inducible gene in PASMCs. In parallel to the upregulation of TERT, the mRNA and protein levels of HIF-1α were also found to increase in response to hypoxia, suggesting that this transcription factor is probably involved in the transcriptional activation of TERT. The increase in HIF-1α mRNA in PASMCs in response to hypoxia indicates that the regulatory response of HIF-1α may occur at the mRNA level.

To further determine the role of HIF-1α in the transcriptional activation of genes in PASMCs, additional experiments were performed to examine the effects of HIF-1α inducers on the expression of TERT genes. It was found that induction of HIF-1α by DFX and $CoCl_2$ significantly increased the HIF-1α mRNA and protein levels and simultaneously increased TERT mRNA. In contrast, inhibition of HIF-1α production by an iron donor, FAS, substantially blocked the hypoxia-induced increase in HIF-1α and TERT mRNA in PASMCs. It is demonstrated that TERT is activated by pharmacologically increased or decreased HIF-1α levels in PASMCs.

Recent studies have demonstrated that ubiquitin-proteasome is a primary protease system responsible for the degradation of HIF-1α, which may importantly contribute to the regulation of intracellular HIF-1α levels and thereby to the transcriptional activation of downstream genes[15-16]. In the present study, TERT transcripts were found to increase markedly even under control conditions, when PASMCs were incubated with a selective inhibitor of ubiquitin-proteasome, CBZ-LLL.This increase in TERT mRNA levels associated with the increase in HIF-1α protein suggests that transcriptional regulation of the TERT gene primarily requires a structural or functional integrity of HIF-1α protein in PASMCs.

In addition to these pharmacological interventions, we also used a molecular decoy approach to determine the role of HIF-1α in the transcriptional regulation of TERT genes. This anti-gene therapy strategy can decoy and thereby block the binding of transcription factors to their binding sites in promoter or enhancer regions by introducing a synthesized dsODN containing a binding cis element[17]. We demonstrated that both hypoxia and HIF-1α induction by $CoCl_2$ increased the mRNA levels of TERT to a much lesser extent in PASMCs transfected with a dsODN containing an HIF-1α binding site, 5'-CGTG-3', compared with control cells. These results provide evidence that specific blockade of HIF-1α binding decreases an increase in TERT mRNA in PASMCs, which further supports our hypothesis that HIF-1α mediates the transcriptional activation of the TERT gene.

In summary, the present studies provided several lines of evidence indicating that TERT expression in PASMCs is transcriptionally regulated via an HIF-1α pathway under normal and hypoxic conditions. First, alterations of HIF-1α formation or degradation by pharmacological interventions changed TERT mRNA levels. Second, the decoy of HIF-1α by transfection of synthesized dsODNs containing 5-CGTG-3 blocked the hypoxia-or $CoCl_2$-induced increase in TERT mRNAlevels. Finally, an in vitro transcription assay demonstrated that hypoxia or induction of HIF-1α increased, but the decoy of HIF-1α decreased, newly formed TERTmRNA in the nuclei of PASMCs.

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