

Full Length Research Paper

Effect of hypoxia-inducible factor 1-alpha (HIF-1 α) on proliferation and apoptosis of adrenocorticotrophic hormone (ACTH)-secreting pituitary adenoma cells

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Accepted 23 May, 2011

To understand whether hypoxia-inducible factor 1-alpha (HIF-1 α) could protect AtT-20 cells from hypoxia induced apoptosis, we investigated the effects of HIF-1 α on proliferation and apoptosis of adrenocorticotrophic hormone (ACTH)-secreting pituitary adenoma cells (AtT-20 cells). AtT-20 cells were treated with various concentrations of CoCl₂ to induce hypoxia. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole (MTT) was applied to detect the proliferation of these cells. Western blot assay and real time polymerase chain reaction (PCR) were used to determine the protein and mRNA expressions of HIF-1 α , respectively. In addition, AtT-20 cells were transfected with siRNA targeting HIF-1 α and treated with different concentrations of CoCl₂. The transfection efficacy was assessed by real-time PCR and western blot assay. Apoptosis was measured by fluorescein isothiocyanate (FITC)-annexin V/ propidium iodide (PI) staining and TUNEL staining. The effect of CoCl₂ on the proliferation of AtT-20 cells was in a concentration and time dependent manner. When the concentration of CoCl₂ was ≤ 100 μ M and/or duration of CoCl₂ treatment was ≤ 48 h, CoCl₂ triggered the proliferation of AtT-20 cells. Nevertheless, the apoptosis rate of cells transfected with HIF-1 α -siRNA was markedly increased after CoCl₂ treatment. These findings suggest that, HIF-1 α can promote the proliferation of AtT-20 cells and exert anti-apoptotic effect under hypoxic condition.

Key words: Hypoxia inducible factor-1 α , cobalt chloride, apoptosis, pituitary adenoma.

INTRODUCTION

Oxygen is indispensable for the growth of solid tumor and it is also true in pituitary adenoma (Shannon et al., 2003). Kristof et al. (2003) demonstrated that the oxygen partial pressure in pituitary adenoma is significantly lower than in the normal pituitary tissues. Hypoxia may promote the apoptosis of tumor cells. However, necrotic or apoptotic cells are seldom found in pituitary adenoma. We speculate that certain factors probably facilitate the tolerance of pituitary adenoma to hypoxia. The expression and activity of hypoxia inducible factor-1 (HIF-1 α) are critical

for the hypoxic accommodation. Our previous study showed HIF-1 α and vascular endothelial growth factor (VEGF), a marker of angiogenesis, which were expressed in different types of pituitary adenoma and their expression levels were positively associated with the invasive property of pituitary adenoma, although, there was no relationship between the expressions of HIF-1 α and VEGF (Zhang et al., 2008). Therefore, HIF-1 α may affect the occurrence and development of pituitary adenoma in a VEGF independent way. In the present study, different concentrations of cobalt chloride (CoCl₂) were employed to induce a hypoxic environment in adrenocorticotrophic hormone-secreting pituitary adenoma cell line (AtT-20 cells) and western blot and real time polymerase chain reaction (PCR) were applied to detect

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the protein and mRNA expressions of HIF-1 α . Then, AtT-20 cells were transfected with siRNA targeting HIF-1 α followed by determination of apoptosis of AtT-20 cells by fluorescein isothiocyanate (FITC)-Annexin V/PI staining and TdT-mediated dUTP-biotin nick end labeling (TUNEL) staining, which further confirmed the role of HIF-1 α in the proliferation of AtT-20 cells under hypoxic conditions.

MATERIALS AND METHODS

Cell culture and CoCl₂ treatment

Mouse AtT-20 cells were kindly provided by Prof. Ning G and Prof. Li XY of Shanghai Institute of Endocrine and Metabolic Diseases. AtT-20 cells have the characteristic of suspended growth and the medium was refreshed every 1 to 2 days. Passaging was performed every 3 to 4 days through centrifugation. Cells were seeded in 6-well plate and incubated with different concentrations (0.25, 50, 100 and 200 μ M) of CoCl₂ in a humidified atmosphere of 95% air and 5% CO₂ at 37°C for 1 to 2 days.

Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole (MTT), dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA), TRIzol (Invitrogen, USA), SYBR®Premix Ex Taq, PCR kit (TaKaRa, Japan), rabbit anti-mouse HIF-1 α polyclonal antibody, mouse specific HIF-1 α -siRNA and mock siRNA (Sigma-Aldrich, USA), Annexin V-FITC apoptosis detection kit (BD, USA) and TUNEL assay kit (Boehringer Mannheim, Germany) were used in the present study.

Detection of proliferation by MTT assay

Cells were seeded in a 96-well plate at a density of 1×10^3 cells/ml followed by incubation with different concentrations of CoCl₂ at 37°C for 24 h. Then, 20 μ l of MTT solution (5 mg/ml) were added to each well followed by incubation for another 4 h. Centrifugation was performed at 2500 rpm for 10 min and the supernatant was removed. The sediment was suspended in 150 μ l of DMSO and stirred in a Vortex mixer for 10 min. The optical density (OD) was measured at 540 nm with a microplate reader.

Extraction of total RNA and real time PCR

Total RNA was extracted with TRIzol and reverse transcription was carried out in a mixture containing 1 μ g of total RNA, 1 μ l of oligo(dT)₁₅ primers and diethylpyrocarbonate (DEPC) treated water at 70°C for 5 min. Then, the mixture was kept on ice for 5 min and mixed with 5 μ l of M-MLV RT 5 \times buffer, 0.5 μ l of 100 mM dNTP, 1 μ l of M-MLV and DEPC treated water (total volume: 15 μ l) followed by reaction at 40°C for 60 min and then 70°C for 15 min. Real time PCR was performed in 20 μ l of mixture with the SYBR®Premix Ex Taq(perfect real time) PCR kit. The conditions for PCR included a pre-denaturation at 95°C for 10 s and 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 31 s. Melt curve was delineated according to the copy number at 65 to 95°C. Data were analyzed with applied biosystems7300 SDS software. $2^{-\Delta\Delta CT}$ was used to calculate the initial copies followed by plotting and statistical analysis. The primers were designed based on the Primer Bank and synthesized in Shanghai Invitrogen. The anticipated length of

products was 228 bp and the primers were as follows: HIF-1 α : forward: 5'-ACCTTCATCGGAACTCCAAAG-3'; reverse: 5'-CTGTTAGGCTGGGAAAAGTTAGG-3'; β -actin: forward: 5'-GGCTGTATTCCCCTCCATCG-3'; reverse: 5'-CCAGTTGGTAAACAATGCCATGT-3'.

Western blot assay

Total protein was extracted and protein concentration was measured with a bicinchoninic acid (BCA) protein assay kit with Bradford method. Then, 100 μ g of protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane followed by blocking in 10% non-fat milk at room temperature for 2 h. The membrane was incubated with rabbit anti-mouse HIF-1 α polyclonal antibody (1:500) at 4°C overnight followed by 3 washes in 1 \times TBST. The membrane was treated with horseradish peroxidase conjugated secondary antibody in TBS containing 5% non-fat milk at room temperature for 1 h. After 3 washes in 1 \times TBST, development was performed with the ECL Plus™ detection system.

Transfection with siRNA

Cells were suspended in antibiotics free medium and 4×10^5 cells were seeded in a 12-well plate flowed by incubation at 37°C in a humidified atmosphere of 5% CO₂ and 95% air for 5 h. When cell confluence reached 90%, these cells were transfected with siRNA targeting HIF-1 α . Briefly, 4 μ l of 10 μ M HIF-1 α -siRNA were mixed with 2 μ l of lipofectamine 2000 and 100 μ l of OPTI-MEM achieving solution A and B, respectively, followed by incubating at room temperature for 5 min. Then, solution A was added to solution B followed by incubation at room temperature for 20 min. The mixture containing solution A and B was added to each well followed by incubation for 24 h. Subsequently, these cells were maintained in antibiotics containing medium and treated with CoCl₂ to induce hypoxia. The transfection efficacy was determined by real time PCR and western blot assay. In addition, cells transfected with mock siRNA served as control and received similar treatment.

Annexin V-FITC staining and TUNEL staining

AtT-20 cells were divided into 4 groups: Group A: normoxia group (control); Group B: CoCl₂ treatment group; Group C: CoCl₂ treatment plus HIF-1 α -siRNA transfection group; Group D: CoCl₂ treatment plus mock siRNA transfection group. Experiment was performed according to instructions.

Statistical analysis

Comparisons of OD values and initial copy number were performed with two-tailed *t* test. Data were expressed as means \pm standard deviation (SD). A value of *P* < 0.05 was considered statistically significant.

RESULTS

Effect of CoCl₂ on AtT-20 proliferation in a time and concentration dependent manner

After treatment with different concentrations (0.25, 50, 100 μ M) of CoCl₂ for 24 h, the proliferation of AtT-20 cells

Table 1. Proliferation of AtT-20 cells after treatment with CoCl₂.

Duration	Concentration					
	0	25 μ M	50 μ M	100 μ M	100 μ M+siRNA	100 μ M+mock
Day 1	0.356 \pm 0.012	0.629 \pm 0.015	0.721 \pm 0.010	0.826 \pm 0.008	0.409 \pm 0.003	0.774 \pm 0.011
Day 2	0.235 \pm 0.015	0.655 \pm 0.023	0.740 \pm 0.006	0.843 \pm 0.007	0.375 \pm 0.005	0.824 \pm 0.006
Day 3	0.124 \pm 0.012	0.472 \pm 0.008	0.554 \pm 0.009	0.711 \pm 0.003	0.218 \pm 0.007	0.682 \pm 0.006

was markedly increased and positively related to the CoCl₂ concentration (Table 1). The highest proliferation was observed at the concentration of 100 μ M. However, treatment with 200 μ M CoCl₂ markedly inhibited the growth of AtT-20 cells (Figure 1a). So, 100 μ M was used as an optimal concentration and treatment was prolonged (a maximum of 3 days) (Figure 1b). But the growth of AtT-20 cells was also significantly suppressed ($P < 0.05$), which may be related to the toxicity of CoCl₂. In addition, after transfection with HIF-1 α -siRNA, cell proliferation was decreased.

mRNA and protein expressions of HIF-1 α

After treatment with different concentrations of CoCl₂ for 24 h, real time PCR and western blot assay were employed to measure the mRNA (Figure 2a) and protein (Figure 2b) expressions, respectively. As shown in Figure 2, expressions of HIF-1 α were detectable in AtT-20 cells under both normoxic and hypoxic conditions, but AtT-20 cells had relatively low expressions of HIF-1 α under normoxic condition. The mRNA and protein expressions of HIF-1 α were increased with the increase in CoCl₂ concentration, which suggests that CoCl₂ treatment can induce hypoxia. When the CoCl₂ concentration was 100 μ M, the expressions of HIF-1 α reached maximal levels. These results were consistent with those in MTT assay.

Transfection efficacy of HIF-1 α -siRNA

AtT-20 cells were divided into 6 groups: Group A: normoxia (control); Group B: normoxia plus mock siRNA transfection; Group C: CoCl₂ treatment; Group D: CoCl₂ treatment plus HIF-1 α -siRNA transfection; Group E: CoCl₂ treatment plus mock siRNA transfection; Group F: AtT-20 cells were incubated in a hypoxia incubator for 24 h. After treatment, real time PCR and western blot assay were performed to measure the mRNA and protein expression of HIF-1 α to determine the transfection efficacy. As shown in Figure 3a, the mRNA expression was down-regulated by >50% ($P < 0.05$), but not markedly changed in the mock siRNA transfection group. Similar trend was also found in the protein expression (Figure 3b). Moreover, our results showed that, incubation in a hypoxia incubator (physical hypoxia) could

also generate similar consequence to that after CoCl₂ treatment (chemical hypoxia).

Apoptosis of AtT-20 cells

FITC-annexin V/PI staining was used to detect the early apoptosis and results showed that, the apoptosis rate was about 10% in CoCl₂ (100 μ M) treatment group and CoCl₂ (100 μ M) treatment plus mock siRNA group. However, transfection with HIF-1 α -siRNA followed by CoCl₂ treatment significantly increased the apoptosis rate (28.90%) ($P < 0.05$). In addition, TUNEL staining (Figure 4) showed that apoptotic cells were only noted in HIF-1 α -siRNA transfection plus CoCl₂ treatment group (Figure 4). These findings suggest that, HIF-1 α can protect the AtT-20 cells against hypoxia induced apoptosis and plays an important role in the occurrence and development of pituitary adenoma.

DISCUSSION

Sufficient blood supply is necessary for the growth of solid tumor and can maintain the essential homeostasis for cell survival. However, when the growth rate of tumor overwhelms that of angiogenesis, the consumption of oxygen and nutrients significantly increases in tumors, resulting in a hypoxic, low glucose and acidic environment. It has been demonstrated that, hypoxia can elicit protective responses to stress. Angiogenesis and anaerobic metabolism are the prerequisites for tumor cells surviving, growing and proliferating in a hypoxic environment. Among numerous factors, the expression and activity of HIF-1 play crucial roles in the hypoxia adaptation of cells. Through regulating gene expression, HIF-1 can promote angiogenesis, hematopoiesis and glycometabolism and may modulate apoptosis, cell cycle and other functions of cells. The pathophysiological effects of HIF-1 are essential for the adaptation of cells to hypoxia (Mabjeesh and Amir, 2007). Evidence shows that, activated HIF-1 can directly or indirectly increase the transcription of about 2% of genes in the human genome (Manalo et al., 2005).

Kristof et al. (2003) reported that, the oxygen partial pressure in pituitary adenoma was significantly lower than in the surrounding normal tissues. Hypoxia may

A

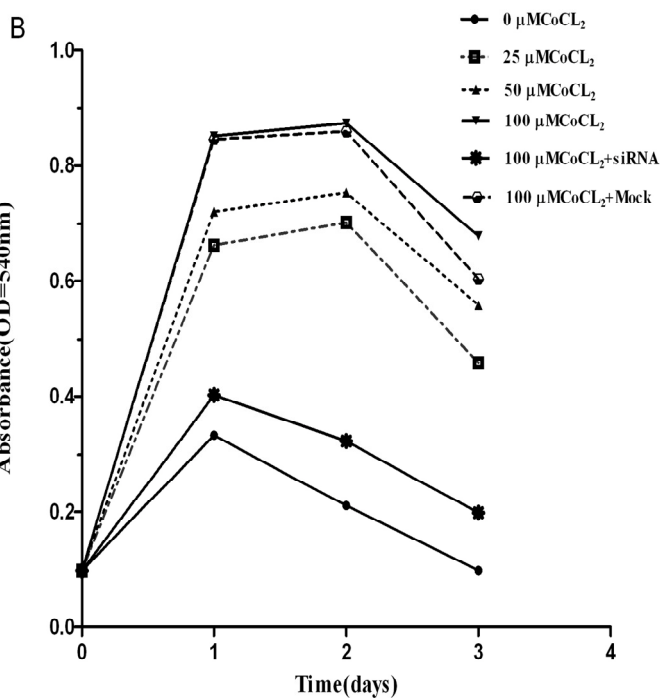
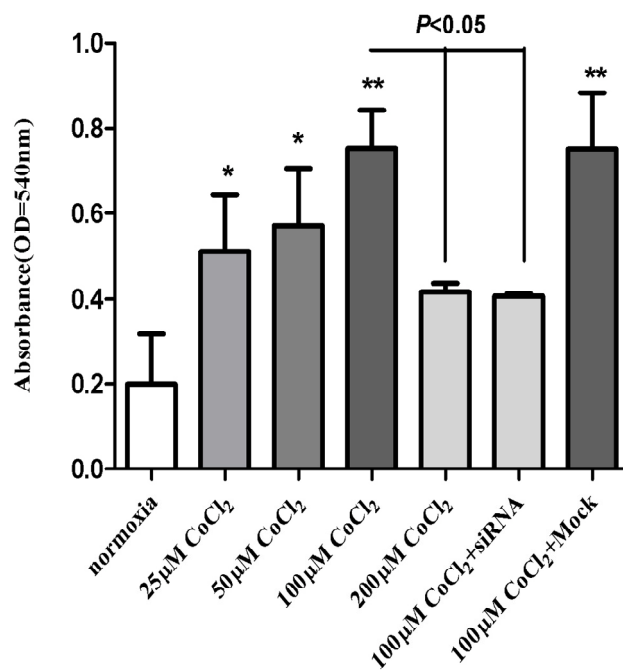


Figure 1. MTT assay was used to detect the proliferation of AtT-20 cells after treatment with different concentrations of CoCl₂. The highest proliferation was observed at the concentration of 100 μM. But 200 μM CoCl₂ resulted in death of a vast majority of AtT-20 cells (A). After treatment with CoCl₂ (≤100 μM) for 1 to 3 days, the proliferation of AtT-20 cells was dramatically decreased ($P < 0.05$). Furthermore, AtT-20 cells transfected with HIF-1 α -siRNA had pronounced decrease in proliferation ($P < 0.05$).

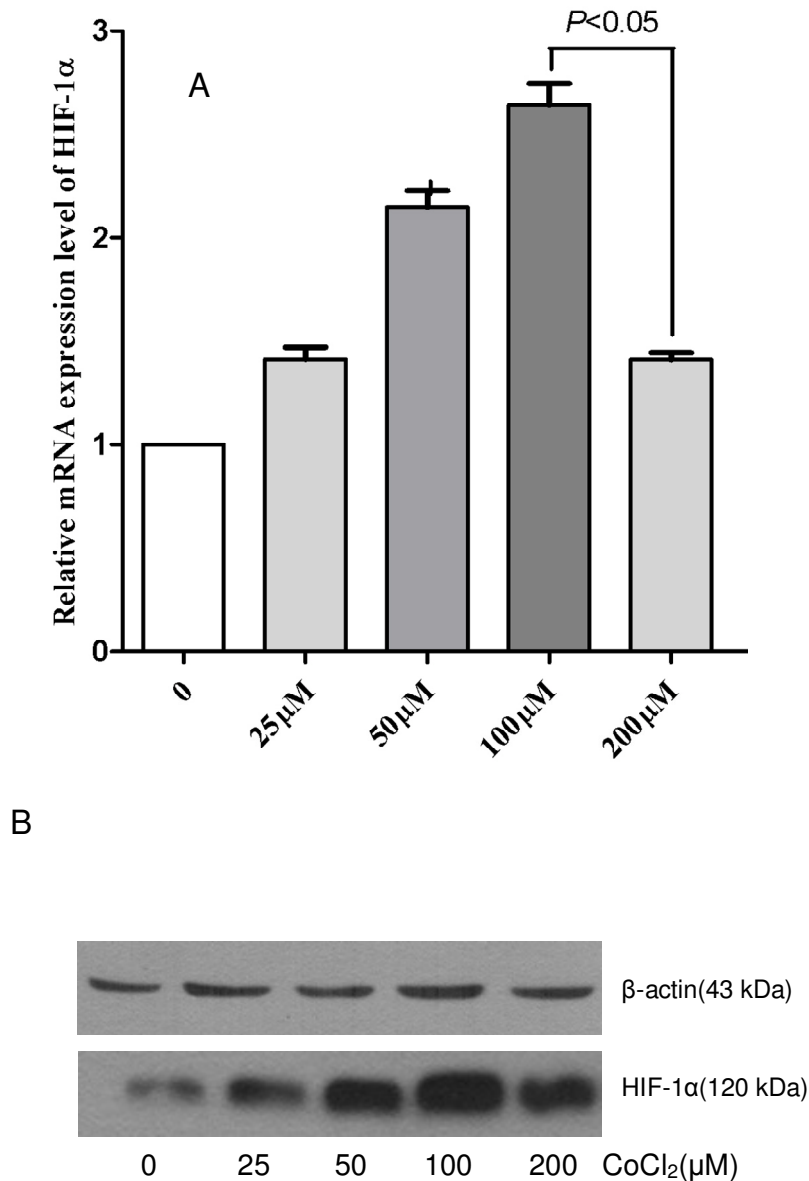


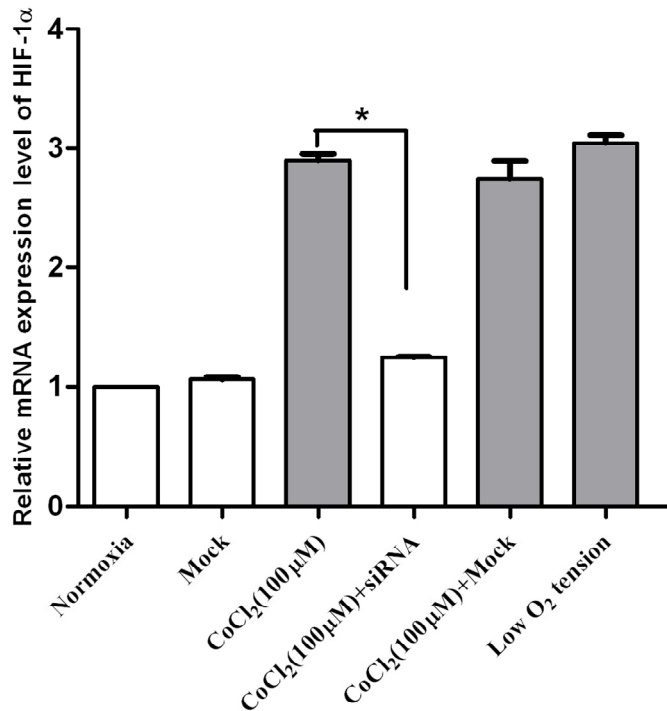
Figure 2. AtT-20 cells were treated with different concentrations of CoCl₂ (0 to 200 μM) for 24 h and real time PCR and western blot assay were employed to detect the mRNA (A) and protein (B) expressions of HIF-1α. Results showed AtT-20 cells had relatively low expressions of HIF-1α under normoxic condition and the expressions of HIF-1α elevated with the increase in CoCl₂ concentration. When the CoCl₂ concentration was 100 μM, the mRNA and protein expression of HIF-1α reached a maximal level. After treatment with 100 μM CoCl₂, the expressions of HIF-1α began to decrease.

facilitate the apoptosis of tumor cells, but necrotic or apoptotic cells were seldom noted in pituitary adenoma. This finding implies that, HIF-1α may be involved in the angiogenesis and apoptosis of pituitary adenoma cells and plays a critical role in the occurrence and development of pituitary adenoma. Vidal et al. (2003) applied immunohistochemistry to detect HIF-1α expression and results showed that, HIF-1α was expressed in a variety of pituitary adenomas. However, the biological role

of HIF-1α in pituitary adenoma is still poorly understood. HIF-1α induced regulatory response can partially explain why the pituitary adenoma cells can survive in a hypoxic environment, but can not explain how these cells proliferate in the absence or insufficiency of nutrients. Escaping from hypoxia induced cell death may be a reasonable explanation.

In the present study, CoCl₂ treatment was conducted to mock a hypoxic environment. Our results showed that,

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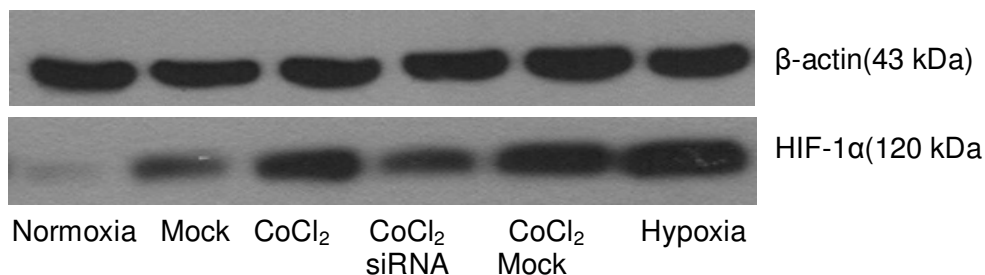


Figure 3. Real-time PCR and western blot assay were employed to detect the transfection efficacy. As shown in A, the mRNA expression of HIF-1 α was decreased by >50% after HIF-1 α -siRNA transfection ($P < 0.05$), but not dramatically reduced after mock siRNA transfection. As shown in A, HIF-1 α -siRNA transfection could also down-regulate the protein expression of HIF-1 α . Furthermore, incubation in a hypoxia incubator for 24 h had similar effects on the HIF-1 α expressions in AtT-20 cells to those after CoCl₂ treatment for 24 h.

treatment with CoCl₂ of different concentrations (0.25, 50, 100 μ M) could promote the proliferation of AtT-20 cells, which was in a concentration dependent manner. The highest proliferation was observed at a concentration of 100 μ M. In addition, 200 μ M CoCl₂ treatment or prolonged CoCl₂ treatment (3 days) could significantly decreased the growth of AtT-20 cells ($P < 0.05$). At the same time, AtT-20 cells were also incubated in a hypoxia incubator to mimic physical hypoxia the effect of which on cell proliferation was compared with that of CoCl₂

treatment serving as chemical hypoxia. Results showed that, incubation in a hypoxia incubator for 24 h could produce similar effect on cell proliferation to that after CoCl₂ treatment at certain concentration for 24 h. Moreover, HIF-1 α -siRNA transfection markedly decreased the proliferation of AtT-20 cells (Figure 1). After HIF-1 α -siRNA transfection followed by CoCl₂ treatment, Annexin V-FITC staining and TUNEL staining (Figure 4) revealed the apoptosis rate was dramatically increased ($P < 0.05$). These findings suggest that, HIF-1 α can protect mouse

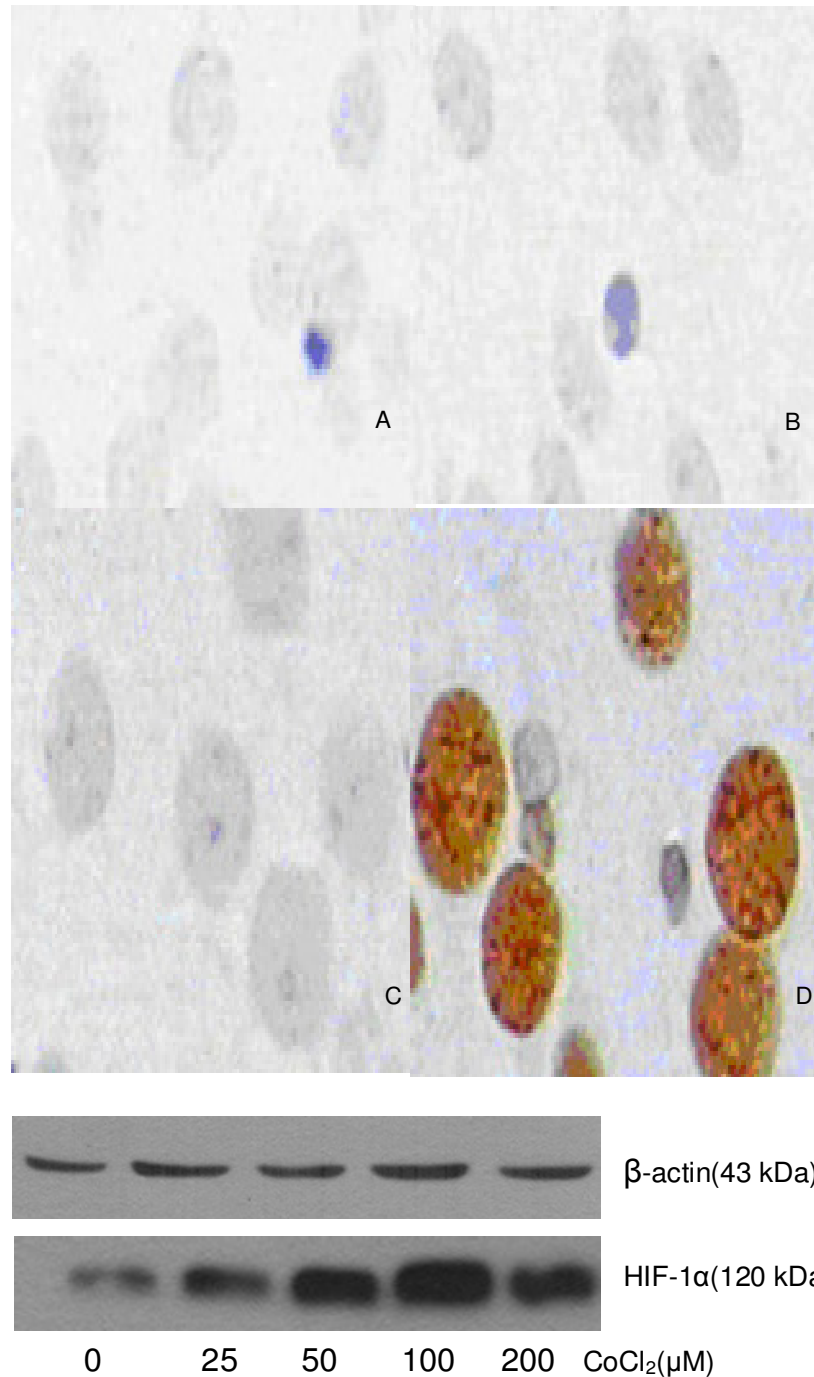


Figure 4. Apoptosis of AtT-20 cells in different groups (TUNEL staining). Apoptotic cells were only observed in the HIF-1 α -siRNA transfection plus CoCl₂ treatment group (4D). A: Normoxia; B: CoCl₂ (100 μ M); C: CoCl₂ (100 μ M) +Mock; D: CoCl₂ (100 μ M)+siRNA.

AtT-20 cells from hypoxia induced apoptosis and plays critical role in the occurrence and development of pituitary adenoma.

Currently, the role of HIF-1 α in apoptosis still remains controversial and conflict results have been reported

(Achison and Hupp, 2003). Study showed that, hypoxia could up-regulate or stabilize proteins of Bcl-2 family to directly exert pro-apoptotic effect (Kothari et al., 2003; Iida et al., 2002; Koshiji et al., 2004; Harris et al., 2002; Ibrahim et al., 2004). Although hypoxia alone can not

induce p53 expression, the p53 expression level is affected by hypoxic microenvironment (Pan et al., 2004). Several studies show that, HIF-1 α can exert protective effect through suppressing hypoxia induced apoptosis (Akakura et al., 2001). Therefore, pancreatic cancer cells expressing HIF-1 α are more tolerant to hypoxia induced apoptosis than similar cells without HIF-1 α expression (Moritz et al., 2002). Evidence shows that, after neutralization of VEGF (a key downstream protein of HIF-1 α) with monoclonal antibody, the anti-apoptotic effect of HIF-1 α is absent, which directly confirms the anti-apoptotic effect of HIF-1 α (Baek et al., 2000). Previous work also reported that, inhibitor of apoptosis protein 2 (IAP-2), an important anti-apoptotic protein, was also regulated by HIF-1 α (Greijer and van der Wall, 2004). Yoshida et al. (2005) applied a microarray analysis technique to detect genomic changes in human nonfunctioning pituitary adenoma cells (HP75 cells) after hypoxia. Their results did not reveal significant changes in expression of p53, Bcl-2 and IAP-2 family. These findings suggest that, the apoptosis related genes regulated by HIF-1 α vary from different tumors. Based on the results of the present study, we speculate that HIF-1 α plays an anti-apoptotic role in the occurrence and development of pituitary adenoma, but the exact biological mechanism should be further studied.

Abbreviations

HIF-1 α , Hypoxia inducible factor-1; **VEGF**, vascular endothelial growth factor; **FITC**, fluorescein isothiocyanate; **TUNEL**, TdT-mediated dUTP-biotin nick end labeling; **MTT**, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole, **DMSO**, dimethyl sulfoxide; **BCA**, bicinchoninic acid; **PVDF**, polyvinylidene fluoride; **SDS-PAGE**, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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