

Propranolol represses infantile hemangioma cell growth through the β 2-adrenergic receptor in a HIF-1 α -dependent manner

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Abstract. Propranolol, as a non-selective blocker of the β -adrenergic receptor (AR), is utilised as the first-line treatment for infantile hemangiomas. However, the underlying mechanism remains poorly understood. The present study was designed to investigate the molecular basis of propranolol on the regression of infantile hemangiomas using a proliferating infantile hemangioma-derived endothelial cell line. In infantile hemangioma patients, we found that propranolol significantly decreased the expression levels of the hypoxia inducible factor (HIF)-1 α in serum and urine, as well as in hemangioma tissues. *In vitro* analysis revealed that propranolol reduces the expression of HIF-1 α in hemangioma cells in a dose- and time-dependent manner, mainly by acting on β 2-AR. Interestingly, it was observed that overexpression of HIF-1 α apparently abrogated the inhibitory effects of propranolol on vascular endothelial growth factor (VEGF) expression and cell growth. Our data further demonstrated that propranolol inhibited the signal transducer and activator of transcription 3 (STAT3), a critical oncogenic signaling molecule, and the anti-apoptotic protein Bcl-2. Additionally, overexpression of HIF-1 α significantly reversed the inhibitory effects of propranolol on STAT3 signaling. In a mouse xenograft hemangioma model, overexpression of HIF-1 α significantly attenuated the therapeutic effects of propranolol and inhibited propranolol-induced hemangioma cell apoptosis. Moreover, the protein levels of VEGF, phosphorylated STAT3, total STAT3 and Bcl-2 were significantly upregulated by HIF-1 α overexpression in propranolol-treated

nude mice bearing hemangiomas. Collectively, our data provide evidence that propranolol may regress infantile hemangiomas by suppressing VEGF and STAT3 signaling pathways in an HIF-1 α -dependent manner.

Introduction

Infantile hemangiomas are a type of benign tumors with high incidence in infancy (1). Typically, the lesions undergo a rapid proliferative phase during early infancy and gradually involute over the first few years (2). Although infantile hemangiomas are benign tumors and usually harmless, they can cause destruction and deformation of facial features, obstruction of breathing and vision, and even more life-threatening complications (3-5). However, the molecular pathogenesis of infantile hemangiomas remains largely unknown and effective treatment still needs to be developed.

Corticosteroids, as well as other drugs such as interferon α and vincristine, have been traditionally regarded as the first-line therapeutic approaches for the treatment of infantile hemangiomas (6-10). However, these drugs are accompanied by multiple and serious side-effects that are very painful for the infants (11-13). Recently, a serendipitous discovery found that propranolol effectively regresses infantile hemangiomas when used to treat obstructive hypertrophic cardiomyopathy accompanied by an infantile hemangioma (14). Propranolol is widely used to treat infantile hemangiomas with satisfactory outcomes and no obviously serious side-effects (15-19). Furthermore, infantile hemangiomas resistant to corticosteroids and interferon or severe infantile hemangiomas may be effectively treated by propranolol (20,21). It has been suggested that propranolol exerts its effects as a non-selective β -adrenergic receptor (AR) blocker that inhibits cell growth and induces cell apoptosis of the endothelial cells (21,22). However, the precise molecular mechanism of its action remains poorly understood.

In recent years, the role of β -AR in tumorigenesis has attracted increasing attention, which is associated with cell proliferation, apoptosis, invasion and metastasis of tumor cells (23). β -AR, including β 1-AR and β 2-AR, are G-protein-coupled receptors on endothelial cells that cause vasodilation of vessels upon activation (24). Moreover, activation of β -AR results in overexpression of the proangiogenic factors, including basic fibroblast growth factor and endothelial

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Abbreviations: AR, β -adrenergic receptor; HIF, hypoxia inducible factor; VEGF, vascular endothelial growth factor; STAT3, signal transducer and activator of transcription 3; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling

Key words: propranolol, infantile hemangiomas, tumorigenesis, hypoxia inducible factor

growth factor (VEGF) and inhibits cell apoptosis (21,22,25). It has been reported that β -AR inhibitors inhibit cell growth and angiogenesis and enhance cell apoptosis of tumors, thereby representing novel antitumor drugs (23). Hypoxia-inducible factor (HIF)-1 consists of two subunits, HIF-1 α and HIF-1 β , and plays an important role in regulating tumor progression (26,27). Of these two subunits, it has been suggested that HIF-1 α is the major regulator for angiogenic factors such as VEGF (27). However, whether propranolol exerts its effects by regulating HIF-1 α -mediated signaling needs to be further investigated.

In the present study, we speculated that β -AR-mediated and HIF-1 α signaling may be associated with the therapeutic effects of propranolol in the treatment of infantile hemangiomas. We found that the elevated HIF-1 α expression levels in infantile hemangioma patients were downregulated by propranolol. Using the hemangioma-derived endothelial cell line, propranolol was found to reduce the expression of HIF-1 α in a dose- and time-dependent manner. Our results further demonstrated that propranolol inhibited HIF-1 α expression through its action on β 2-AR. Moreover, we revealed that propranolol suppressed cell growth by inhibiting HIF-1 α -VEGF signaling. Additionally, the signal transducer and activator of transcription 3 (STAT3) and Bcl-2, which are critical oncogenic signaling molecules, were found to be increased in infantile hemangiomas, whereas propranolol was found to inhibit STAT3 and Bcl-2 expressions in a HIF-1 α -dependent manner. Overexpression of HIF-1 α attenuated the therapeutic effects of propranolol on hemangiomas in a mouse model, which further confirmed that propranolol regressed infantile hemangiomas in a HIF-1 α -dependent manner. Collectively, we represent a potential mechanism of propranolol in which propranolol represses infantile hemangioma cell growth by inhibiting VEGF, STAT3 and Bcl-2 expression in a HIF-1 α -dependent manner, which leads to cell growth arrest and the induction of cell apoptosis.

Materials and methods

Sample collections. A total of 11 infantile hemangioma patients were recruited in the present study from June 2012 to May 2013 at the Second Affiliated Hospital of Xi'an Jiaotong University. The patients were orally treated with propranolol (Tianjin Lisheng Pharmaceutical Co., Ltd., Tianjing, China). The patients had received no surgery or drug treatments prior to the propranolol treatment. Before and after the propranolol treatment, venous blood, urine and tumor tissues (d=2 mm) were collected with the informed consent of the family members and the approval of the Ethics Committee of the Second Affiliated Hospital of Xi'an Jiaotong University.

Animals and cell culture. Six-week-old female BALB/c nude mice (20-30 g) were provided by the Experimental Animal Centre of the College of Medicine of Xi'an Jiaotong University. The mice were housed in a standard pathogen-free room according to the standard feeding protocols and the animal experimental procedures were handled in accordance with the Institutional Animal Care and Use Committee of Xi'an Jiaotong University. The hemangioma-derived endothelial cell line from proliferating infantile hemangioma tissues

was previously prepared and established in our laboratory (28). The cell line was cultured in a RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10 ng/ml epidermal growth factor and 15% fetal bovine serum (FBS) plus 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified atmosphere chamber containing 5% CO₂ at 37°C.

Enzyme-linked immunosorbent assay (ELISA). The collected blood and urine samples were centrifuged at 1,000 x g for 15 min. The supernatants were collected and the concentration of HIF-1 α was measured using an ELISA reagent kit (R&D Systems, Minneapolis, MN, USA) as per the supplier's instructions and analyzed by an ELISA reader (BioTek Instruments Inc., Winooski, VT, USA).

Cell treatments and transfections. For propranolol treatment, hemangioma endothelial cells were treated with various concentrations of propranolol (0, 10, 50 and 100 μ M) and incubated for 24, 48 and 72 h. For gene overexpression or gene knockdown, recombinant lentiviral vectors (Shanghai GenePharma Co., Ltd., Shanghai, China) or specific siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were transfected with the cells and incubated for 48 h prior to being collected for analysis.

Western blot analysis. Total proteins were extracted from tumor tissues or cells and quantified using a BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Approximately 25 μ g protein was run on a precast 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electro-blotting onto a nitrocellulose membrane (Amersham, Little Chalfont, UK). The membranes were blocked by blocking buffer (3% skimmed milk solution) at 37°C for 1 h. The membranes were then blotted with primary antibodies diluted in blocking buffer overnight at 4°C. After being washed three times with Tris-buffered saline (TBS) and Tween (TBST) (each for 5 min), the membranes were incubated with horseradish peroxidase conjugated secondary antibody (Wuhan Boster Biological Technology, Ltd., Wuhan, China) in the blocking buffer for 1 h. The membranes were then washed three times with TBST and once with TBS, and the blots were developed with an enhanced chemiluminescence (ECL) detection system (Amersham). The following primary antibodies were used: anti-HIF-1 α , anti-VEGF, anti-STAT3, anti-p-STAT3, anti- β 1-AR, anti- β 2-AR, anti-Bcl-2 and anti-GAPDH (Santa Cruz Biotechnology).

MTT assay. Cell growth was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cells were grown in 96-well plates (1x10⁴ cells/well) and cultured until they reached 80% confluence. Thereafter, the cells were treated with HIF-1 α , siRNA or LV-HIF-1 α with 50 μ M propranolol and incubated for 48 h. MTT diluted in PBS (5 mg/ml) was added at 20 μ l/well and continually incubated for 4 h. Dimethylsulfoxide (150 μ l/well) was added to dissolve the formazan crystals. The optical density value was measured using an ELISA reader at 490 nm.

Mouse xenograft experiment. A xenograft of hemangioma cells in the mice was prepared as previously described (28).

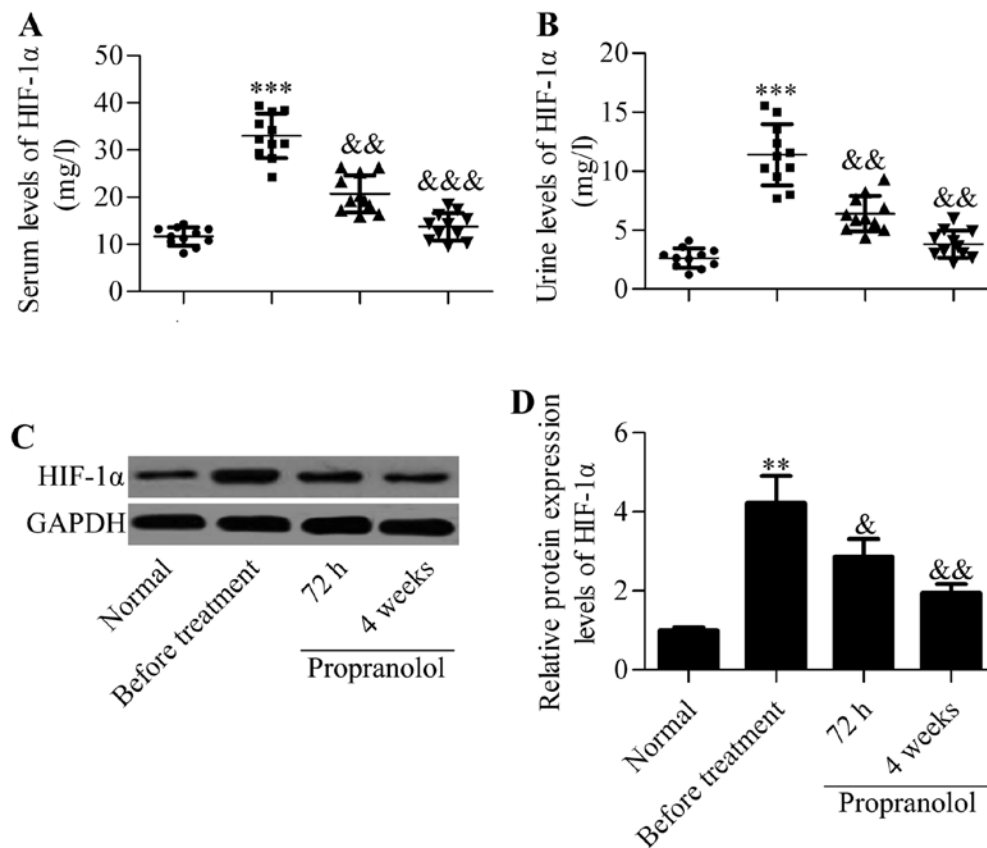


Figure 1. Expression levels of HIF-1 α in infantile hemangioma patients treated with propranolol. Detection of HIF-1 α concentrations in serum (A) and urine (B) by ELISA methods. Serum and urine samples were collected from normal, healthy subjects that were taken as a control; before treatment, infantile hemangioma patients before propranolol treatments; 72 h or 4 weeks, infantile hemangioma patients treated with propranolol for 72 h or 4 weeks. N=11, ***P<0.001 vs. normal, &&&P<0.001 and &&P<0.01 vs. before treatment. (C) Detection of HIF-1 α protein levels in hemangioma tissues. Normal, vascular tissues from surgical operations; hemangioma tissues were isolated from infantile hemangioma patients before or after propranolol treatments for 72 h or 4 weeks. (D) Quantification of relative protein levels of HIF-1 α normalized to GAPDH using Image-Pro Plus 6.0 software. N=5, **P<0.01 vs. normal, &P<0.05 and &&P<0.01 vs. before treatment.

Briefly, the cells (4×10^{10}) that were diluted in 200 μ l PBS were injected subcutaneously into the right groin. Two days after the tumor cell implantation, recombinant lentivirus (5×10^{10} plaque-forming units) in 200 μ l PBS was injected subcutaneously into the left groin and this was performed every two weeks. The nude mice were intragastrically administered with propranolol solution (0.25 mg/ml) at 0.2 ml/10 g body weight every two days. After 40 days, the mice were euthanized by subcutaneous injection with sodium pentobarbital (40 mg/kg) and tumor tissues were harvested for analysis.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Cell apoptosis was detected by a TUNEL staining assay using a one-step TUNEL apoptosis assay kit (Beyotime, Haimen, China). Briefly, tumor tissues were dissected, fixed with 4% paraformaldehyde for 24 h at 4°C, gradient-dehydrated, embedded in paraffin and then serially cut into 5 μ m-thick sections. The sections were dewaxed, gradient-rehydrated and digested with 20 μ M proteinase for 10 min. After being washed with PBS, the apoptotic cells were examined using a TUNEL apoptosis assay kit according to the supplier's instructions. The sections were observed under a fluorescence microscope (Olympus, Tokyo, Japan) and the TUNEL staining cells were calculated in five random fields and averaged per field.

Statistical analysis. The data were expressed as mean \pm standard deviation (SD). Statistical analyses were performed using one-way ANOVA followed by the Bonferroni post hoc test among multiple groups. A value of P<0.05 was regarded as statistically significant.

Results

HIF-1 α is downregulated by propranolol treatment in infantile hemangioma patients. To explore the molecular basis of propranolol in the treatment of infantile hemangioma, we evaluated the effect of propranolol on the levels of HIF-1 α in infantile hemangioma patients. By using ELISA detection methods, we found that the concentrations of HIF-1 α in the serum and urine were significantly increased in infantile hemangioma patients compared with those from healthy subjects. Surprisingly, the serum and urine levels of HIF-1 α were significantly decreased after treatment with propranolol (Fig. 1A and B). To further validate that propranolol regulated the expression of HIF-1 α , we detected the expression of HIF-1 α in the hemangioma tumor tissues by western blot analysis. Similar results were obtained showing that propranolol treatment significantly decreased the protein levels of HIF-1 α , which had been upregulated in infantile hemangioma tissues without treatment (Fig. 1C and D). Collectively, the results

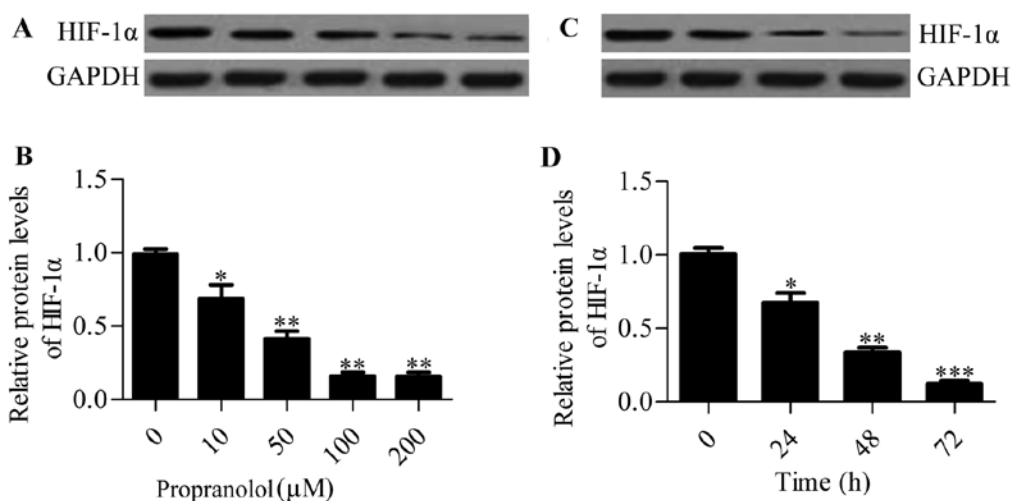


Figure 2. Effect of propranolol on the expression of HIF-1 α in hemangioma cells *in vitro*. (A) Detection of HIF-1 α protein expression in cell groups treated at different doses, by western blot analysis and (B) representative histograms showing quantification of relative protein levels. The cells were treated with various concentrations of propranolol (10, 50, 100 and 200 μ M) for 48 h. N=3, *P<0.05, **P<0.01 vs. 0 μ M. (C) Detection of HIF-1 α protein expression in 100 μ M propranolol-treated cells for 24, 48 and 72 h by western blot analysis, and (D) representative histograms showing quantification of relative protein levels. N=3, *P<0.05, **P<0.01 and ***P<0.001 vs. 0 h.

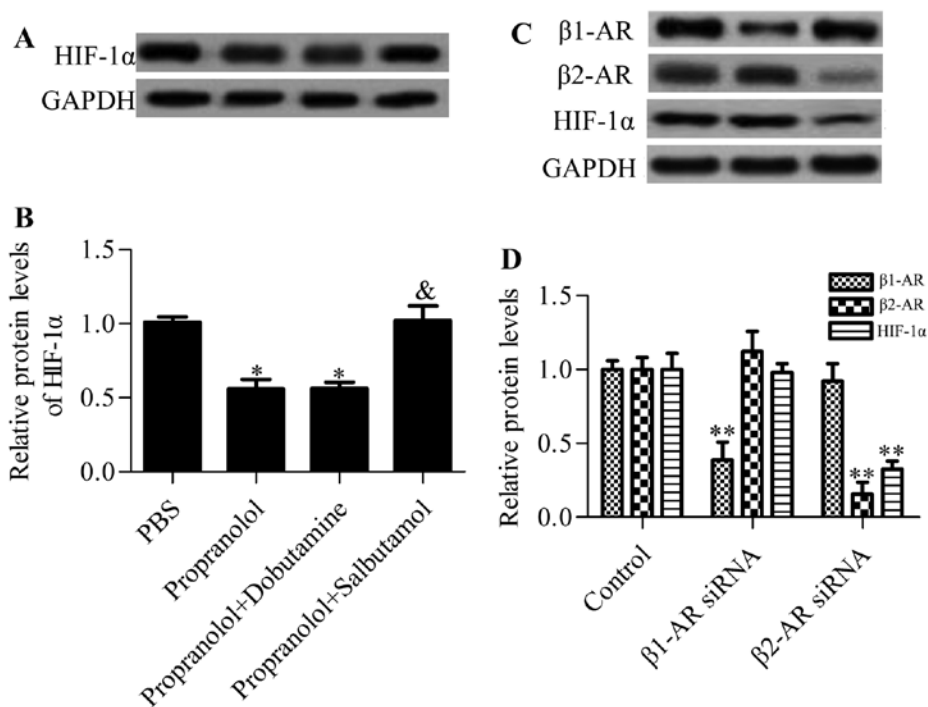


Figure 3. The role of β -AR in propranolol-induced HIF-1 α inhibition. (A) Detection of the β 1-AR agonist (dobutamine) or β 2-AR agonist (salbutamol) in propranolol-induced HIF-1 α inhibition by western blot analysis, and (B) representative histograms showing quantification of relative protein levels. Dobutamine (10 μ M) or salbutamol (100 nM) were added to propranolol-treated cells for 48 h. N=3, *P<0.05 vs. PBS; &P<0.05 vs. propranolol. (C) Detection of the effects of β -AR knockdown on HIF-1 α expression by western blot analysis, and (D) representative histograms showing quantification of relative protein levels. Non-specific siRNA was the control. N=3, **P<0.01 vs. control.

suggested that propranolol inhibited HIF-1 α in infantile hemangioma patients.

Propranolol reduces the expression of HIF-1 α in infantile hemangioma cells in a dose- and time-dependent manner. To further verify the regulated effect of propranolol on HIF-1 α expression, we used the hemangioma-derived endothelial cell line to investigate the effect of propranolol on HIF-1 α expres-

sion *in vitro*. Hemangioma endothelial cells were treated with varying concentrations of propranolol (0, 10, 50, 100 and 200 μ M) for 48 h and the HIF-1 α protein expression was determined by western blot analysis. The results showed that propranolol administration decreased the HIF-1 α expression in a dose-dependent manner (Fig. 2A and B). Next, we treated the cells with 100 μ M propranolol and incubated them for 24, 48 and 72 h (Fig. 2C and D). The results showed that HIF-1 α

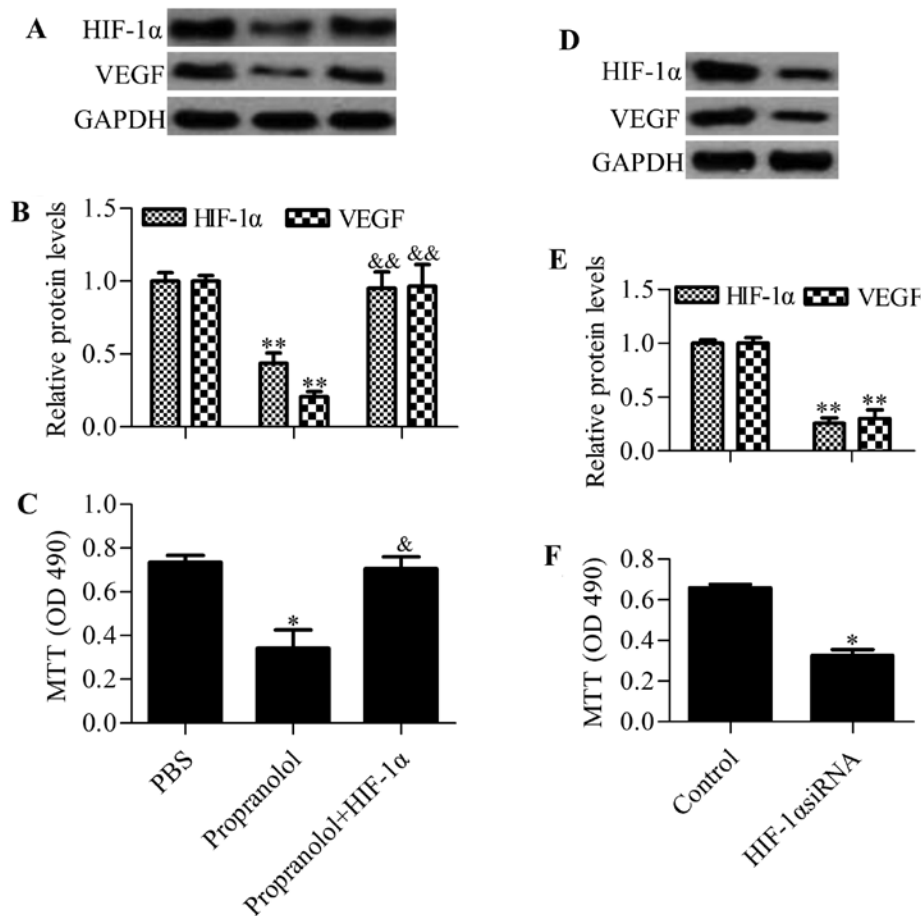


Figure 4. The role of HIF-1 α in propranolol-mediated cell growth of hemangioma cells. (A) Detection of HIF-1 α overexpression on VEGF expression in propranolol-treated cells by western blot analysis, and (B) representative histograms showing quantification of relative protein levels. N=3, **P<0.01 vs. PBS, &P<0.01 vs. propranolol. (C) An MTT assay to detect the effect of HIF-1 α overexpression on propranolol-mediated cell growth. N=5, *P<0.05 vs. PBS, &P<0.05 vs. propranolol. (D) Detection of HIF-1 α knockdown on VEGF expression by western blot analysis, and (E) representative histograms showing quantification of relative protein levels. N=3, **P<0.01 vs. control. (F) An MTT assay was performed to detect the effect of HIF-1 α knockdown on cell growth. N=5, *P<0.05 vs. control.

expression was increased at 24 h and continuously increased up to 72 h. In conclusion, these results further indicated that propranolol regulated the expression of HIF-1 α in the infantile hemangioma.

Propranolol inhibits the expression of HIF-1 α mainly through acting on the β 2-adrenergic receptor (AR). Propranolol is a non-selective antagonist for β 1-AR and β 2-AR (21). To determine which β -AR played an important role in mediating the inhibitory effect of propranolol on the expression of HIF-1 α , we added the β 1-AR agonist (dobutamine) or the β 2-AR agonist (salbutamol) to propranolol-treated cells and examined their effects on the expression of HIF-1 α . The results showed that the β 1-AR agonist had no apparent effect on the propranolol-induced HIF-1 α decrease, whereas the β 2-AR agonist significantly reversed the inhibitory effects of propranolol on HIF-1 α expression (Fig. 3A and B). Additionally, inhibition of β 2-AR by specific β 2-AR siRNA had the same effect as propranolol on HIF-1 α . However, knockdown of β 1-AR had no apparent effect on HIF-1 α expression (Fig. 3C and D). Collectively, these results suggested that propranolol repressed the expression of HIF-1 α mainly through acting on β 2-AR, not β 1-AR.

Overexpression of HIF-1 α abrogates the inhibitory effects of propranolol on hemangioma cell growth. To gain insight into HIF-1 α in propranolol-induced cell growth arrest, we examined the effect of HIF-1 α overexpression on propranolol-treated cells. The results showed that propranolol treatment inhibited the protein expression of HIF-1 α and VEGF, whereas overexpression of HIF-1 α blocked the inhibitory effects of propranolol on VEGF expression (Fig. 4A and B). Furthermore, propranolol-induced cell growth arrest was also abrogated by HIF-1 α overexpression (Fig. 4C). Similarly, knockdown of HIF-1 α resulted in a decreased VEGF expression (Fig. 4D and E) and cell growth inhibition (Fig. 4F), which had the same effect as propranolol treatment. These results implied that propranolol suppressed hemangioma cell growth by regulating HIF-1 α .

Propranolol inhibits the expression of STAT3 and Bcl-2 in hemangioma cells. STAT3, a critical molecule for regulating oncogenic signaling, has been previously reported to be overexpressed in proliferating infantile hemangiomas (29). To investigate whether propranolol had an inhibitory effect on the expression of STAT3, we detected the protein expression of STAT3 in propranolol-treated hemangioma cells by western

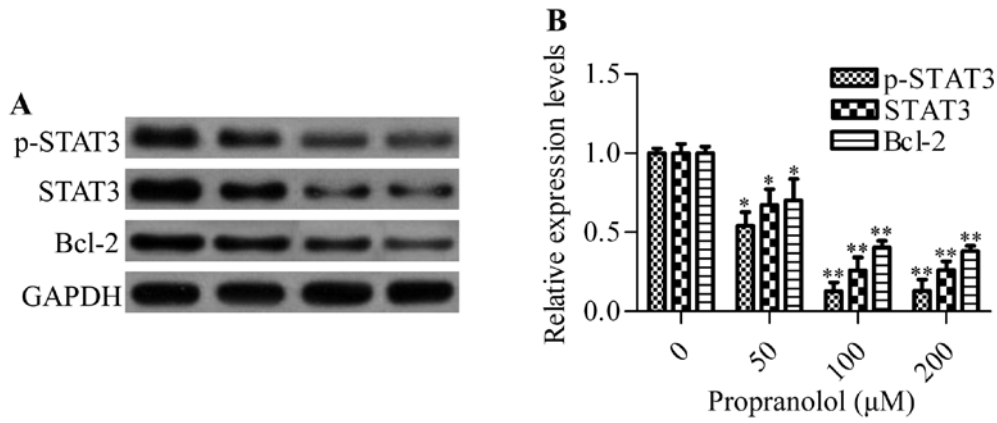


Figure 5. Effect of propranolol on STAT3 signaling. (A) Detection of different concentrations of propranolol (0, 50, 100 and 200 μ M) in the expression of p-STAT3, total STAT3 and Bcl-2 in hemangioma cells. (B) Representative histograms showing quantification of relative protein levels. N=3, *P<0.05 and **P<0.01 vs. 0 μ M.

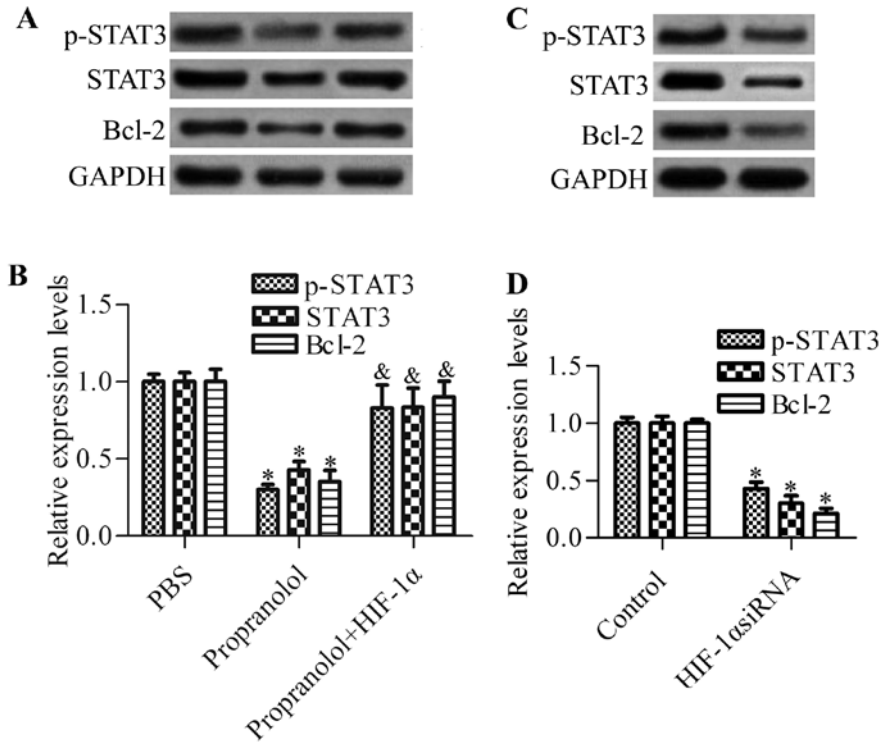


Figure 6. The role of HIF-1 α in propranolol-inhibited STAT3 signaling. (A) Detection of HIF-1 α overexpression in the protein expression of p-STAT3, total STAT3 and Bcl-2 by western blot analysis. (B) Representative histograms showing quantification of the relative protein levels. N=3, *P<0.05 vs. PBS, &P<0.05 vs. propranolol. (C) Detection of HIF-1 α knockdown in STAT3 signaling by western blot analysis, and (D) representative histograms showing quantification of the relative protein levels. N=3, *P<0.05 vs. control.

blot analysis. The results showed that different concentrations of propranolol significantly inhibited the expression of total STAT3 and phosphorylated STAT3 (p-STAT3). Furthermore, the downstream gene Bcl-2, an anti-apoptotic gene, was also markedly decreased by propranolol (Fig. 5). These data indicated that propranolol was capable of inhibiting STAT3 signaling.

Overexpression of HIF-1 α abrogates the inhibitory effects of propranolol on STAT3 signaling. To investigate whether HIF-1 α was involved in the regulation of STAT3 signaling,

we detected the effect of HIF-1 α overexpression on STAT3 signaling activation in propranolol-treated cells by western blot analysis (Fig. 6A). The results showed that overexpression of HIF-1 α significantly abrogated the inhibitory effects of propranolol on the protein expression of p-STAT3, STAT3 and Bcl-2 (Fig. 6B). Knockdown of HIF-1 α by siRNA markedly decreased the protein expression of p-STAT3, STAT3 and Bcl-2, which mimics the effect of propranolol on STAT3 signaling. Collectively, these results suggested that HIF-1 α played an important role in propranolol-mediated inhibition of STAT3 signaling in hemangioma cells.

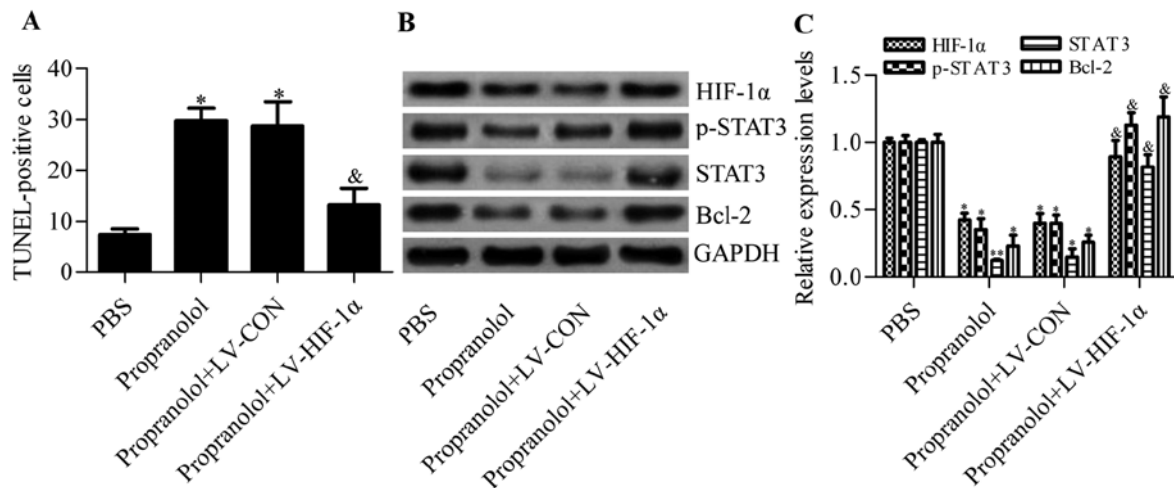


Figure 7. Detection of the effects of HIF-1 α overexpression on the therapeutic effects of propranolol on a mouse xenograft hemangioma model. (A) Quantitative analysis of TUNEL-positive cells in tumor tissues from different treated groups. PBS, nude mice bearing hemangioma tumors treated with PBS; propranolol, nude mice bearing hemangioma tumors treated with propranolol; propranolol+LV-CON, nude mice bearing hemangioma tumors were treated with propranolol and control LV-CON; propranolol+HIF-1 α , nude mice bearing hemangioma tumors were treated with propranolol and LV-HIF-1 α . N=5, *P<0.05 vs. PBS; &P<0.05 vs. propranolol. (B) Detection of the effects of HIF-1 α overexpression on the protein levels of VEGF, p-STAT3, STAT3 and Bcl-2 in tumor tissues by western blot analysis, and (C) representative histograms showing quantification of relative protein levels. N=3, *P<0.05, **P<0.01 vs. PBS; &P 0.05 vs. propranolol.

Overexpression of HIF-1 α reduces the therapeutic effects of propranolol on hemangiomas in a mouse model. To further validate the important role of HIF-1 α involved in propranolol treatment for hemangiomas, we infected a mouse xenograft hemangioma model with LV-HIF-1 α overexpressing HIF-1 α . Using the TUNEL method, we found that propranolol-induced hemangioma apoptosis was apparently inhibited by HIF-1 α overexpression (Fig. 7A). Furthermore, the protein expression of VEGF, p-STAT3, STAT3 and Bcl-2, downregulated by propranolol in tumor tissues, was significantly upregulated by HIF-1 α overexpression (Fig. 7B and C). Collectively, these results further confirmed that propranolol repressed hemangiomas in a HIF-1 α -dependent manner.

Discussion

Although the use of propranolol has been broadly applied in the clinical treatment of infantile hemangiomas, the underlying mechanism remains largely unknown. Therefore, it is of great importance to gain better insight into propranolol in the treatment of infantile hemangiomas to enable better patient care as well as to contribute to the development of novel therapies. In the present study, we sought to delineate the molecular mechanism of propranolol in the treatment of infantile hemangiomas. Furthermore, evidence was presented that propranolol represses infantile hemangioma cell growth through its action on β 2-AR and inhibits VEGF, STAT3 and Bcl-2 expression in an HIF-1 α -dependent manner, which leads to cell growth arrest and the induction of cell apoptosis using a hemangioma endothelial cell model.

Formerly, propranolol was generally used for the treatment of cardiovascular diseases including hypertension, supraventricular tachycardia, ischemic heart disease and arrhythmia, and has been proved to be safe and well tolerated (30). In 2008 Léauté-Labrèze *et al* (14) used propranolol to treat obstructive hypertrophic cardiomyopathy accompanied by

infantile hemangiomas and unexpectedly found that propranolol effectively regresses infantile hemangiomas. Since then, an increasing number of studies have reported the use of propranolol in the treatment of infantile hemangiomas (31-33). Currently, propranolol is regarded as the first-line drug for the treatment of infantile hemangiomas due to its capacity to provide instant gratification and be more effective, and its few side-effects, as well as its low cost (34-36). However, the underlying mechanism is still elusive. In recent years, numerous studies have been devoted to investigating the molecular basis of propranolol in the treatment of infantile hemangiomas. Some possible mechanisms have been proposed, including vasoconstriction, decreased expression of VEGF and the triggering of apoptosis (22). Nonetheless, there is still a lack of precise understanding.

β -AR has been reported to be expressed in various tumor cells and the activation of β -AR results in an increase in the cyclic AMP (cAMP) that activates the cAMP-dependent protein kinases and the multiple signaling pathways (37-39). Many studies have demonstrated that activation of β -AR exhibits the tumor-promoting function in various tumor cells (40,41). β -AR antagonists have been found to inhibit cell proliferation, invasion and migration of the tumor cells (42-44). Propranolol has been reported as a non-selective β -AR blocker of β 1-AR and β 2-AR (21). In the present study, we found that propranolol inhibited cell growth of hemangioma endothelial cells mainly through its action on β 2-AR, and knockdown of β 2-AR had the same effect as propranolol on cell growth, while knockdown of β 1-AR had no apparent effect on cell growth. Our results indicated that propranolol repressed infantile hemangiomas by inhibiting β 2-AR-mediated signaling pathways. Consistently, Truong *et al* (45) reported that β 2-AR expression and phosphorylation responded to propranolol treatment of infantile hemangiomas.

HIF-1 α has been proposed as an important mediator in regulating tumor progression (26,27). In the present study, we

found that HIF-1 α was upregulated in the serum, urine and tumor tissues in infantile hemangioma patients, and treatment of propranolol markedly inhibited the expression levels of HIF-1 α . The results also implied that HIF-1 α was involved in regulating infantile hemangiomas. Our data further identified that propranolol suppressed HIF-1 α by inhibiting β 2-AR. The link between HIF-1 α and β -AR has been reported in a variety of tumors, including breast, prostate and pancreatic cancer cells (46,47). β -AR agonists have been shown to increase HIF-1 α expression, and β -AR agonist-induced HIF-1 α and VEGF expression was abrogated by propranolol (47). More recently, propranolol was suggested to repress hemangioma cells by downregulating HIF-1 α , VEGF and downstream signaling pathways (48). The present study presented evidence that propranolol blocks β 2-AR leading to decreased HIF-1 α and VEGF expression, which may explain the molecular basis of propranolol action. Furthermore, our data demonstrated that propranolol inhibited the expression and phosphorylation of STAT3 in infantile hemangioma cells. The overexpression of STAT3 has been reported in infantile hemangiomas (29) and angiosarcomas (49).

In summary, our results suggested that propranolol repressed infantile hemangioma cell growth through its action on β 2-AR and inhibits VEGF, STAT3 and Bcl-2 expression leading to cell growth arrest and the induction of cell apoptosis using a hemangioma endothelial cell model. Propranolol treatment also decreased the expression of HIF-1 α , which is a critical regulator of tumor progression. Overexpression of HIF-1 α significantly abrogated the effects of propranolol, implying that propranolol regressed infantile hemangiomas in a HIF-1 α -dependent manner. These findings suggested a plausible mechanism of propranolol in the regression of infantile hemangiomas and provided novel insights for the development of novel therapeutic methods for infantile hemangiomas.

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