

## Original Article

# Propranolol induces apoptosis in endothelial cells by inhibiting Akt and ERK phosphorylation and MAPK signaling pathway

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Received February 1, 2017; Accepted July 28, 2017; Epub September 15, 2017; Published September 30, 2017

**Abstract:** Background: Propranolol has been used for treatment of infantile hemangioma. This study is to investigate the effect and underlying biological mechanism of propranolol on infantile hemangioma cell growth. Method: Different concentrations of propranolol (10, 25, 50 and 100  $\mu$ M, respectively) was used to treat endothelial cells (ECs), meanwhile, the control group was propranolol free. The proliferation of ECs was detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Moreover, effects of propranolol on cells cycle and apoptosis were detected by flow cytometry. Key proteins in protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) signaling pathway were detected by Western-Blot and cAMP levels were measured by cAMP enzyme-linked immunosorbent assay (ELISA) kit. Results: Propranolol inhibited the proliferation of ECs and induced G0/G1 blockade in a concentration-dependent manner. In addition, our results elucidated that propranolol induced apoptosis of ECs and this pro-apoptotic effect was shown to be concentration-dependent. Furthermore, propranolol induced apoptosis by inhibiting Akt and ERK phosphorylation and MAPK signaling pathway. Conclusion: Propranolol could inhibit the proliferation of ECs by inhibiting Akt and ERK phosphorylation.

**Keywords:** Propranolol, infantile hemangioma, endothelial cell, MAPK, apoptosis

## Introduction

Infantile hemangioma (IH) is one of the common benign tumors in children [1-3]. Although most hemangiomas can be self-limiting, they rapidly invade important tissues and organs, hence resulting in complications such as ulceration, bleeding, and infection. Therefore, its potential harm to infants and young children cannot be ignored. The incidence rate of IH is nearly 10%, but 4 to 5 percent is probably a better estimate [4]. IH often occurs in the superficial location of the head, face and limbs, which consists of mitotic endothelial cells (ECs) and interstitial components. It develops fast with the EC proliferation and angiogenesis (proliferative phase) in the first few months of babies. Until the infants are 1 years old, it begins to spontaneously subside [5, 6]. Although IH becomes dissipated at 5-10 years old, it is important to pay attention to its potential hazards. The current treatment for IH contains sur-

gical resection, oral steroids, local injection of pingyangmycin, isotope applicator, and laser treatment [1, 6, 7]. However, there are some limitations of these treatments. Emerging studies reported 2-3 mg/(kg.d) propranolol could significantly inhibit IH [8]. As side effects of propranolol are relatively few and there is no death or serious cardiovascular events reported in children so far, propranolol has been considered to be the preferred IH strategy.

Léauté-Labréze *et al.* show that propranolol treatment on children had prominent effects, such as thinner tumor, lighter color and surface shrinkage, indicating that it improves IH by changing the tumor blood vessel hemodynamics and reducing local blood supply [8]. Currently, the biological mechanism of propranolol inhibiting IH is controversial. Existing views can be summarized to vasoconstrictor effect [9], induce apoptosis of ECs through the  $\beta$ -adrenergic receptor signaling pathway [10,

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11], caspase signaling pathway [12, 13] and inhibit angiogenesis by down-regulating vascular endothelial growth factor and basic fibroblast growth factor (bFGF) [11, 14, 15]. Previous studies have shown that propranolol inhibits the migration of endothelial progenitor cells (EPCs) by influencing Akt and mitogen-activated protein kinase (MAPK) signaling pathways [16]. Akt and MAPK signaling pathways are the classical downstream of the  $\beta$ -ADR signal, which play important roles in cell proliferation, differentiation, apoptosis, and migration [17, 18]. In addition, MAPK contains extracellular regulated protein kinases (ERK), p38 and c-Jun N-terminal kinase (JNK), of which ERK1/2 is closely related to cell proliferation. When ERK1/2 pathway is significantly inhibited, it leads to the inactivation of anti-apoptotic B-cell lymphoma 2 (Bcl-2), thus accelerating the process of apoptosis [19].

This study was to investigate the effect of propranolol on proliferation, cells cycle, apoptosis, Akt and MAPK signal pathways, as well as its underlying mechanism in ECs with IH. Our results suggested that propranolol induced apoptosis in endothelial cells by inhibiting Akt and ERK phosphorylation and MAPK signaling pathway, thus, it was beneficial for IH.

### Materials and methods

#### *Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay*

All cells were incubated at 37°C and 5% CO<sub>2</sub> atmosphere for 1 day before the study began. ECs were digested, counted, and then plated evenly at a concentration of 8000 cells/well in a 96-well plate (200  $\mu$ l/well). The medium was removed and 100  $\mu$ l fresh medium containing different concentrations of propranolol was added, with 3-5 wells in each treatment. Then 10  $\mu$ l 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide was added. After the supernatant was carefully removed, purple crystals were dissolved in 150  $\mu$ l dimethylsulfoxide (DMSO)/well and lysed for 10 min at 500 rpm in a vortex mixer. The cell viability was measured at 490 nm with a full-wave microplate reader.

#### *Flow cytometry*

The cells were treated with 0.125% trypsin, centrifuged, and washed twice with cold phosphate buffer saline (PBS). ECs were suspended

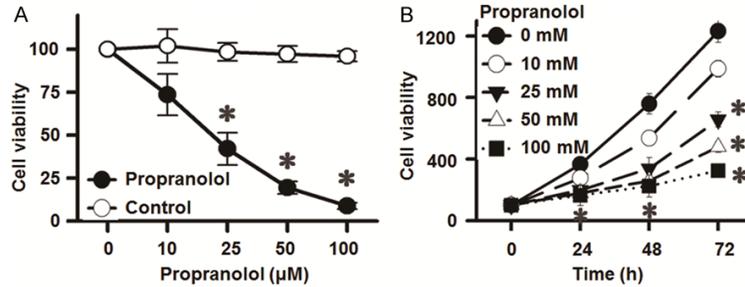
in 0.25 ml cold PBS to form a single cell suspension. Subsequently, 0.75 ml of ice-cold ethanol was slowly added dropwise, mixed well and fixed at -20°C for 24 hours, followed by centrifuged at 1500 rpm for 5 min, removed ethanol and washed twice with PBS. After cells passed through a 300 mesh sieve (70  $\mu$ m), they were suspended in 0.5 ml PI stain at 4°C. Then the result was detected by flow cytometry.

Additionally, the apoptosis of ECs induced by propranolol was also detected by flow cytometry. The cells were digested with trypsin without ethylene diamine tetraacetic acid (EDTA) and then the cell count was collected. ECs ( $1-5 \times 10^5$ ) were suspended in 500  $\mu$ l binding buffer and mixed with 5  $\mu$ l Annexin V-FITC in the dark for 10 min. After 5  $\mu$ l PI was added, they were mixed and incubated for 5-15 min in the dark at room temperature. The cells were detected within one hour by flow cytometry according to the manufacturer's protocol.

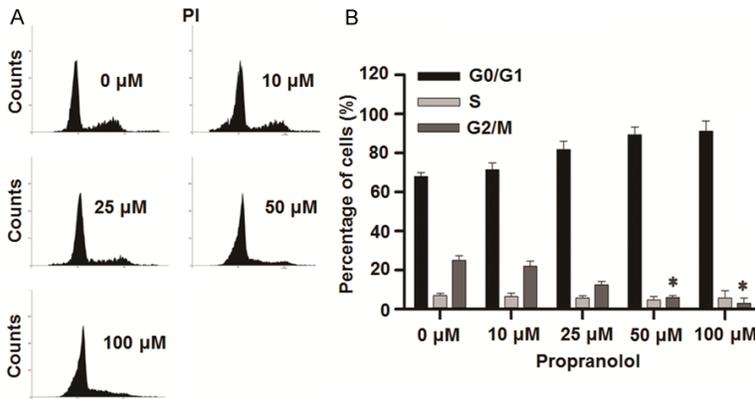
#### *Western-blot*

Phosphorylated proteins were extracted from propranolol-treated cells using a cell lysate containing a phosphatase inhibitor, followed by configuring with 12% separation gel and 4% concentration of plastic gel. Protein samples were mixed with 2  $\times$  loading buffer at a volume ratio of 1:1 and boiled at 100°C for 5 min. Then 30-40  $\mu$ g protein samples were used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis condition is for the concentration of plastic gel 45 V about 30 min and separation gel 120 V about 2-3 hours. The protein was transferred to a polyvinylidene fluoride (PVDF) membrane using a Bio-Rad wet transfer system for 120 min. Then 5% skimmed milk was used as blocking agent and primary antibodies of mouse anti-human Akt and ERK (1:1000, Sigma-Aldrich, St. Louis, MO, USA) were added. After incubation with the membrane at 4°C overnight, Tris buffered saline tween (TBST) was added to wash the unbound primary antibody 3 times, each time for 10 minutes. Then diluted HRP labeled secondary rabbit anti-mouse antibody (1:2000, Thermo Fisher Scientific, Waltham, MA, USA) was added and incubated at room temperature for 1 hour. Enhanced chemiluminescence was used to expose protein band. The protein bands were grey scale analyzed by Bio-Rad Discovery software (Quantity One). B-actin was taken as the reference.

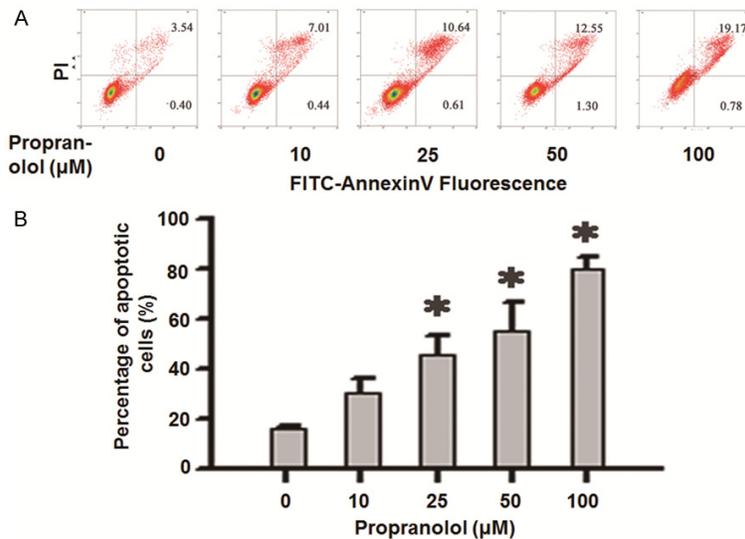
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**Figure 1.** Propranolol inhibited the proliferation of ECs. A. Effects of different concentrations of propranolol on cell viability; B. Effects of different time of propranolol treatments on cell viability. EC, endothelial cell. \*,  $P < 0.05$ .



**Figure 2.** Flow cytometry was used to analyze the effect of propranolol on cell cycle. A. Propranolol treatment resulted in a G0/G1 cell cycle arrest in ECs; B. Propranolol treatment reduced the S phase of ECs. EC, endothelial cell. \*,  $P < 0.05$ .



**Figure 3.** Flow cytometry was used to analyze propranolol-induced apoptosis in ECs. A. Effects of propranolol treatment on ECs apoptosis; B. The apoptotic cells in ECs by propranolol treatment. EC, endothelial cell. \*,  $P < 0.05$ .

### cAMP assay

Cells were co-incubated with adrenaline (100 μM) or propranolol (25 μM) for 1 h in culture medium and cAMP levels were measured by cAMP ELISA kit (Cell Biolabs)-competitive enzyme-linked immunosorbent assay strictly according to the manufacturer's protocol.

### Statistical analysis

All data were analyzed for variance by SPSS 19.0 statistical software (SPSS Inc, Chicago, IL, USA). Results were represented as mean  $\pm$  standard deviation (SD). Differences were considered significant if  $P < 0.05$ .

## Results

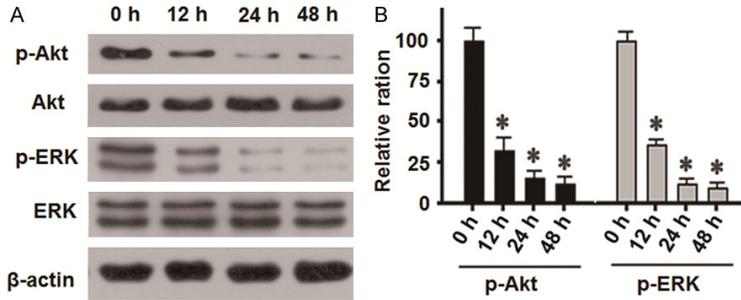
### Propranolol inhibited the proliferation of ECs

EC proliferation is an important process in angiogenesis. To investigate whether propranolol was effective on EC proliferation, ECs were incubated with 10, 25, 50, and 100 μM of propranolol for 72 h, respectively. As shown in **Figure 1**, the proliferation of ECs was significantly inhibited by propranolol. The inhibitory effect was positively correlated with the concentration of propranolol and time (**Figure 1**). These data suggested that propranolol could specifically inhibit the proliferation of ECs.

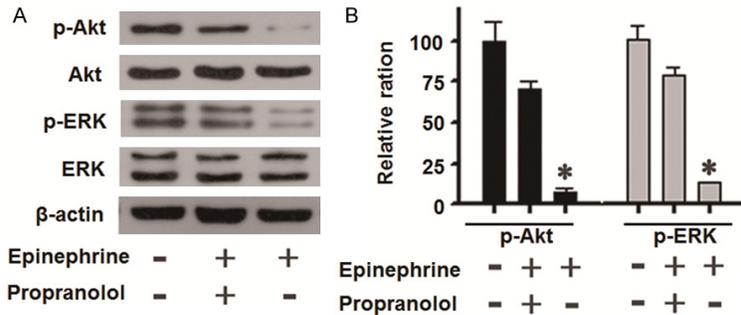
### Propranolol induced G0/G1 blockade in ECs

To further explore the mechanism of propranolol inhibition on cell proliferation, the DNA content in ECs with or

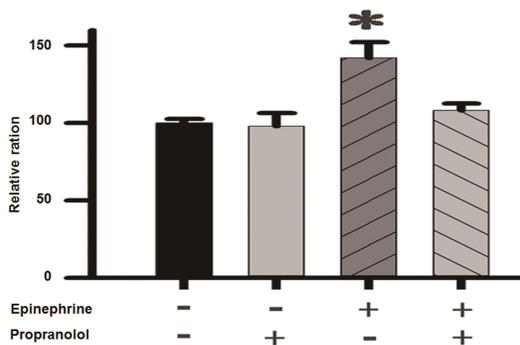
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**Figure 4.** Propranolol inhibited Akt and ERK signaling pathway. Akt, protein kinase B; ERK, extracellular signal-regulated kinase. \*,  $P < 0.05$ .



**Figure 5.** Propranolol inhibited the effect of epinephrine by upregulation of Akt and ERK phosphorylation. Akt, protein kinase B; ERK, extracellular signal-regulated kinase. \*,  $P < 0.05$ .



**Figure 6.** The effect of propranolol on intracellular cAMP. \*,  $P < 0.05$ .

without propranolol treatment was detected by flow cytometry, and the effect of propranolol on cell cycle was examined. As shown in **Figure 2A**, propranolol-treated ECs had a potent G0/G1 cell cycle arrest with a significant reduction in S and M phases compared with that of the control group. Propranolol treatment for 20 h effectively reduced the S phase of the cells (**Figure 2B**). The results indicated that propranolol affected the transition from G1 phase to S

phase of the cell cycle in ECs and exhibited a concentration-dependent manner.

### *Propranolol induced apoptosis of ECs*

To investigate whether propranolol can cause apoptosis in ECs, apoptosis was analyzed by PI and Annexin V staining. The percentage of apoptotic cells increased gradually as drug concentration increased, showing a concentration-dependent pattern compared with the drug-free control group (**Figure 3A** and **3B**). This result indicated that propranolol treatment promoted EC apoptosis.

### *Propranolol inhibited Akt and MAPK phosphorylation*

$\beta$ -adrenergic receptors are widely expressed on the EC membrane. As a non-selective  $\beta$ -blockers, propranolol can compete with  $\beta$ -adrenergic receptor to antagonize  $\beta$ -adrenergic effect [17]. The ERK and Akt signaling pathways, downstream of the  $\beta$ -adrenergic receptor, are closely related to the processes of cell proliferation, differentiation, migration and apoptosis [18, 19]. Therefore, we examined the phosphorylation of Akt and ERK by Western-blot. As shown in **Figure 4**, propranolol significantly inhibited Akt and ERK phosphorylation in a time-dependent manner.

Propranolol may inhibit the phosphorylation of Akt and ERK through  $\beta$ -ADR pathway. Therefore, we treated ECs with high concentrations of epinephrine (100  $\mu$ M) and propranolol (20  $\mu$ M) to observe whether propranolol can inhibit up-regulated Akt and ERK phosphorylation by adrenaline. The results showed that the phosphorylation level of cells treated with both drugs and without drug treatment was significantly higher than that of cells treated with propranolol alone (**Figure 5**). Our results suggested that propranolol inhibited the effects of epinephrine on up-regulation of Akt and ERK phosphorylation via the  $\beta$ -ADR pathway.

cAMP is an important molecule in the MAPK signaling pathway and the increased intracellular cAMP concentration will activate the MAPK pathway [20]. Thus, we examined the cAMP concentration of propranolol-treated cells, as shown in **Figure 6**, it is found that the level of cAMP in propranolol-treated ECs was similar to that of the drug-free control group. Intracellular cAMP levels significantly reduced by both drugs compared to the cells treated with epinephrine alone. These data indicated that propranolol could affect cell apoptosis through MAPK signaling pathway.

### Discussion

Recent studies have shown that propranolol can affect the proliferation, differentiation and migration of ECs through  $\beta$ -ADR signal transduction pathway. In the present study, the data demonstrated propranolol inhibited the proliferation of ECs with IH. Our findings were in agreement with previous reports that propranolol could inhibit hemangioma-derived endothelial cells (HemECs) proliferation and induce apoptosis [21, 22]. Furthermore, our results also suggested that propranolol affected the transition from G1 to S phase in ECs and exhibited a concentration-dependent pattern, which was consisted with previous reports [23, 24].

Apoptosis is a process of programmed cell death, which is characterized by nuclear condensation and fragmentation, cellular shrinkage without loss of plasma membrane integrity and the formation of apoptotic bodies. Apoptosis is strictly regulated by a complex interplay of caspases, pro-caspases and proteins of the Bcl-2 family. It has been reported that propranolol caused significant apoptosis of ECs in IH and other diseases [12, 13, 21]. Our results further elucidated that propranolol induced apoptosis of ECs in IH and the proportion of apoptosis cells increased significantly. Moreover, this pro-apoptotic effect was shown to be concentration-dependent.

In the present study, we also investigated the mechanism of propranolol-induced apoptosis of ECs in IH. Akt is a serine/threonine protein kinase, which is released from the plasma membrane and exerts its anti-apoptotic effect by phosphorylating target proteins in a variety of downstream pathways. For example, Akt can prevent apoptosis by phosphorylating Bcl-2

family members BAD, and inhibiting the activity of proteolytic enzymes caspase-9. ERK and Akt signaling pathways play important roles in the anti-apoptotic process of cells. Apoptosis may be induced by down-regulating phosphorylation of ERK and Akt. Propranolol may affect migration of EPCs through Akt and MAPK pathways, but not the proliferation of EPCs. EPCs are a kind of immature ECs that can be further proliferated and differentiated into ECs with migration characteristics. ECs and EPCs differ in the type of  $\beta$ -ADR expressed on the cell surface [25]. Activation of ERK1/2 was translocated to the nucleus and activated by P90RSK, MSK and transcription factors ELK-1, Stat3 phosphorylation, which caused cell growth, proliferation and differentiation. ERK activated the cell cycle progression by promoting the expression of CyclinD1 and combined with cyclin-dependent kinase 4 (CDK4), leading to cell proliferation or differentiation. The continued activation of ERK could prevent the occurrence of apoptosis. The down-regulation of ERK activity may lead to cell cycle arrest and cell apoptosis. Activation of Akt plays an anti-apoptotic effect through phosphorylation of a variety of downstream factors, such as enzymes, kinase and transcription factors. Propranolol may play an important role in the apoptosis of ECs in IH by down-regulation of Akt phosphorylation. In the present study, we demonstrated that propranolol induced apoptosis by inhibiting Akt and ERK phosphorylation and MAPK signaling pathway. Our findings were in agreement with the previous studies that explored the mechanisms of propranolol action in IH [26, 27]. Our data also could provide the new possible treatment of propranolol in other human neoplasms.

In a summary, our results for the first time described the molecular mechanism of propranolol-induced EC apoptosis in IH and also confirmed the important roles of AKT, ERK and MAPK in angiogenesis. Propranolol inhibited the proliferation and induced G0/G1 blockade of ECs in IH in a concentration-dependent pattern. In addition, our data elucidated that the propranolol induced apoptosis of ECs in IH and this pro-apoptotic effect was shown to be concentration-dependent. Furthermore, propranolol induced apoptosis by inhibiting Akt and ERK phosphorylation and inhibiting MAPK signaling pathway. The outcome suggested propranolol had the anti-angiogenic effects. So, it could be

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an attractive potential therapeutic target not only for IH, but also for solid tumors.

## Acknowledgements

This study was supported by Natural Science Foundation of Shandong Province (ZR2015-HL012).

## Disclosure of conflict of interest

None.

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