



Propranolol suppresses angiogenesis *in vitro*: Inhibition of proliferation, migration, and differentiation of endothelial cells

Sylvie Lamy, Marie-Paule Lachambre, Simon Lord-Dufour, Richard Béliveau *

Laboratoire de Médecine Moléculaire, Université du Québec à Montréal, C.P. 8888, Succ. Centre-ville, Montréal, Québec, Canada H3C 3P8

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ABSTRACT

Propranolol, a non-selective β -adrenergic blocking drug, was recently reported to control the growth of hemangiomas, the most common vascular tumor of infancy. However, the mechanisms involved in this effect remain unknown. Here, we demonstrate that propranolol dose-dependently inhibited growth factor-induced proliferation of cultured human umbilical vein endothelial cells (HUVECs) through a G_0/G_1 phase cell cycle arrest. This was correlated to decreased cyclin D1, cyclin D3, and cyclin-dependent kinase CDK6 protein levels, while increases in the CDK inhibitors p15^{INK4B}, p21^{WAF1/Cip1} and p27^{Kip1} were observed. Chemotactic motility and differentiation of HUVECs into capillary-like tubular structures in Matrigel were also inhibited by propranolol. Furthermore, inhibition by propranolol of vascular endothelial growth factor (VEGF)-induced tyrosine phosphorylation of VEGF receptor-2 lead to inhibition of downstream signaling such as the activation of the extracellular signal-regulated kinase-1/2 and the secretion of the extracellular matrix degrading enzyme MMP-2. Taken together, these results demonstrate that propranolol interferes with several essential steps of neovascularization and opens up novel therapeutic opportunities for the use of β -blockers in the treatment of angiogenesis-dependent human diseases.

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1. Introduction

Angiogenesis, the process of new blood vessel growth from pre-existing capillaries, is essential for embryogenesis, functioning of the female reproductive system, and wound healing (Carmeliet and Jain, 2000). An imbalance in this process contributes to the pathogenesis of numerous disorders such as diabetic retinopathy, chronic inflammation, tumor growth and metastasis (Folkman, 1995a). Excess angiogenesis occurs through a series of steps, including stimulation of endothelial cells (ECs) by autocrine and/or paracrine growth factors, proteolytic degradation of the basement membrane and surrounding extracellular matrix, EC migration and proliferation, and structural reorganization into a three-dimensionally tubular structure (Liekens et al., 2001). Thus, the suppression of abnormal angiogenesis

may provide therapeutic strategies for the treatment of angiogenesis-dependent disorders.

Beta-adrenoceptor antagonists (β -blockers) are one of the most widely used classes of drugs in cardiovascular diseases as well as in the management of anxiety, migraine and glaucoma (Lowenthal et al., 1984; Reiter, 2004). Propranolol, a non-selective β -blocker, was one of the first successful β -adrenergic receptor (β -AR) blocking agents developed for clinical use (Mak and Weglicki, 2004). It specifically competes with β -AR agonists such as epinephrine and norepinephrine at the β_1 - or β_2 -AR sites (Reiter, 2004). Adrenoceptors are located in a wide variety of tissues and tumors (Reiter, 2004), and catecholamines are potent direct stimulators of the migration of various human carcinoma cell types (Masur et al., 2001; Thaker et al., 2006; Yang et al., 2006) as well as of the secretion of proangiogenic factors by these tumors (Chakraborty et al., 2009; Yang et al., 2006). Indeed, previous studies have shown a reduced cancer risk for patients taking β -blockers (Algazi et al., 2004; Perron et al., 2004). Moreover, it has been reported that propranolol can inhibit the norepinephrine-driven metastasis development of human cancer cells in nude mice (Palm et al., 2006; Sood et al., 2006). Recently, propranolol was shown to inhibit the growth of capillary hemangiomas in children who, in fact, received the drug for cardiac complications during corticosteroid therapy (Leaute-Labreze et al., 2008). The authors hypothesized that the therapeutic effect of propranolol was due to vasoconstriction, decreased expression of the genes for vascular endothelial growth factor (VEGF) and basic

Abbreviations: β -AR, beta-adrenergic receptor; bFGF, basic fibroblast growth factor; CDK, cyclin-dependent kinase; CKI, cyclin kinase inhibitor; EC, endothelial cell; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GPCR, G-protein-coupled receptor; HUVEC, human umbilical vein endothelial cell; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; RTK, receptor tyrosine kinases; Tyr(P), phosphotyrosine; VEGF, vascular endothelial growth factor; VEGFR-2, vascular endothelial growth factor receptor-2.

* Corresponding author. Laboratoire de Médecine Moléculaire, Département de Chimie, Université du Québec à Montréal, C.P. 8888, Succ. Centre-ville, Montréal, Québec, Canada H3C 3P8. Tel.: +1 514 987 3000x8551; fax: +1 514 987 0246.

E-mail address: oncomol@nobel.si.uqam.ca (R. Béliveau).

fibroblast growth factor (bFGF) (which contribute to angiogenesis) and to the triggering of apoptosis in capillary ECs. However, the mechanisms underlying the inhibition of angiogenesis by propranolol remain to be established.

The present study investigated whether the β -blocker propranolol can modulate EC functions essential for angiogenesis. We observed that propranolol inhibited growth factor-induced proliferation, migration, and the morphogenic differentiation of EC into capillary-like structures *in vitro*. We also found that propranolol caused G₁ cell cycling arrest of EC through the modulation of the cyclin-dependent kinase (CDK) and cyclin kinase inhibitor of CDK (CKI) machinery, and the inhibition of VEGF-induced VEGF receptor-2 (VEGFR-2) signaling pathways. Taken together, these novel activities of propranolol shed light on the antiangiogenic mechanisms involved, and highlight its potential therapeutic application against angiogenesis-dependent diseases.

2. Materials and methods

2.1. Materials

Cell culture media were obtained from Life Technologies (Burlington, ON) and serum was purchased from HyClone Laboratories (Logan, UT). Matrigel basement membrane matrix was obtained from Becton Dickinson Labware (Bedford, MA). Norepinephrine, propranolol hydrochloride and other chemical reagents, unless stated otherwise, were obtained from Sigma-Aldrich (Oakville, ON). Human recombinant VEGF was obtained from R&D Systems (Minneapolis, MN). PTK787 was obtained from Novartis Pharmaceuticals (Basel, Switzerland). The MEK kinase inhibitor U0126 was from Calbiochem (La Jolla, CA). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). Protein A-Sepharose and Protein G-Sepharose were obtained from Amersham Pharmacia Biotech (Baie d'Urfé, Qc). The anti-VEGFR-2 (C-1158) and anti-ERK-1/2 (extracellular signal-regulated kinase 1 and 2) (K-23) polyclonal antibodies and the anti-phosphotyrosine (anti-Tyr(P)) (PY99) monoclonal antibody were from Santa Cruz Biotechnologies (Santa Cruz, CA). Antibodies for cyclin D1, cyclin D3, CDK4, CDK6, p21 monoclonal antibodies, and p15, p21, p27 and anti-phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr 202/Tyr 204) polyclonal antibodies were from Cell Signaling Technology (Beverly, MA). Anti-mouse and anti-rabbit horseradish peroxidase-linked secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and enhanced chemiluminescence (ECL) reagents were from PerkinElmer Life Sciences (Boston, MA). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HMVECs-d-Ad) were purchased from Clonetics (San Diego, CA). Cells were maintained in EC basal medium-2 (EBM-2; Lonza, Walkersville, MD) supplemented with 2% fetal bovine serum (FBS) for HUVECs and 5% FBS for HMVECs and with EGM-2 growth factor mixture (Lonza). Cells used in this study were restricted to use between passages 3 and 6. They were cultured at 37 °C under a humidified 95%–5% (v/v) mixture of air and CO₂. Cells were treated with vehicle or with propranolol diluted in 100% EtOH and stimulated with growth factors or VEGF.

2.3. Cell proliferation assays

HUVECs were plated in 96-well plates at 8000 cells/well in 200 μ L complete medium and incubated at 37 °C under a humidified atmosphere containing 5% CO₂ for 24 h. Then, the medium was removed and replaced by 100 μ L of fresh medium containing 5% FBS and the indicated concentrations of propranolol. Cell proliferation was deter-

mined by assaying the mitochondrial activity of cells, after a total of 30 h incubation, using the highly sensitive WST-1 (water-soluble tetrazolium salt) assay. Briefly, 10 μ L of WST-1 reagent (Roche, Laval, QC) was added to each well and the soluble formazan dye produced by metabolically active cells was monitored every minute for 40 min at 37 °C on a SpectraMax Plus reader (Molecular Devices, Sunnyvale, CA) at 450 nm.

2.4. Cytotoxicity assays

To assess the effect of propranolol on cell viability, the release of lactate dehydrogenase (LDH) upon damage of the plasma membrane was analyzed in the culture medium (containing 5% FBS) of HUVECs. Samples from the cell medium were harvested from cells treated with propranolol for 30 h. LDH activity was measured at 30 °C by a continuous optical test based on the extinction change of pyridine nucleotide at 340 nm as described by the manufacturer's instructions (Promega).

2.5. Cell cycle analysis

HUVECs were incubated in medium containing the indicated concentration of propranolol supplemented with 5% FBS for 6–30 h. At the end of treatment, the cells were collected by mild trypsin digestion, washed with ice-cold phosphate-buffered saline (PBS)/ethylenediaminetetraacetic acid (EDTA) (5 mM) and then resuspended in 1 volume of PBS/EDTA and fixed/permeabilized in 100% ethanol overnight. Three volumes of staining solution, containing propidium iodide (PI, 50 μ g/mL) and DNase-free RNase (20 μ g/mL), were added for at least 30 min at 37 °C before analysis. The fraction of population in each phase of the cell cycle was determined as a function of the DNA content using a BD Biosciences FACS Calibur flow cytometer equipped with CellQuest Pro software.

2.6. Western blot analysis for cell cycle-related proteins

HUVECs were treated as described for cell cycle analysis and solubilized in lysis buffer [150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM ethyleneglycol-O, O'-bis(2-aminoethyl)-N, N, N', N'-tetraacetic acid (EGTA), 0.5% (vol/vol) Nonidet P-40 and 1% (vol/vol) Triton X-100]. Lysates (20 μ g) were solubilized in Laemmli sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% β -mercaptoethanol, and 0.00125% bromophenol blue], boiled for 4 min, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes which were then blocked overnight at 4 °C with 5% nonfat dry milk in Tris-buffered saline/Tween 20 (TBS-T; 147 mM NaCl, 20 mM Tris-HCl, pH 7.5, and 0.1% Tween 20). Membranes were further washed in TBS-T and incubated with the primary antibody (anti-cyclin D1, anti-cyclin D3, anti-CDK4, anti-CDK6, anti-p15, anti-p21 or anti-p27) in TBS-T containing 3% bovine serum albumin (BSA), followed by a 1 h incubation with HRP-conjugated anti-mouse antibodies in TBS-T containing 5% nonfat dry milk. Immunoreactive material was visualized with an ECL detection system.

2.7. Endothelial cell tube formation assays

Matrigel (12.5 mg/mL) was thawed at 4 °C and 50 μ L was quickly added to each well of a 96-well plate and allowed to solidify for 10 min at 37 °C. Once solid, the wells were incubated for 30 min with 100 μ L of cells (20,000 cells/well) in EBM-2. After adhesion of the cells, the indicated concentrations of propranolol was added in the medium and the wells were incubated at 37 °C for 6 h (HUVECs) and 18 h (HMVECs). The formation of capillary-like structures was examined microscopically and pictures (50 \times) were taken using a

Retiga 1300 camera and a Zeiss Axiovert S100 microscope. The extent to which capillary-like structures formed in the gel was quantified by analysis of digitized images to determine the thread length of the capillary-like network, using a commercially available image analysis program (Northern Eclipse).

2.8. Migration assays

Transwells (8- μ m pore size; Costar) were precoated with 0.15% gelatin in PBS by adding 600/100 μ L in the lower/upper chambers followed by overnight incubation at 4 °C. The Transwells were then washed with PBS and assembled into 24-well plates. The upper chamber of each Transwell was filled with 50 μ L of cells (10^6 cells/mL) and HUVECs were allowed to adhere for 30 min. The monolayers were then treated for 2 h by adding 50 μ L of 2-fold concentrated propranolol solution prepared in serum-free medium into the upper chamber and 600 μ L of the compound solution (1 \times) into the lower chamber. After 2 h, cell migration was initiated by adding growth factor-enriched media derived from the human U87 glioblastoma cell line to the lower chamber. The plate was placed at 37 °C in 5%CO₂/95% air for another 4 h. Cells that had migrated to the lower surface of the filters were fixed and stained, and migrations were then quantified as described previously (Lamy et al., 2006a).

2.9. Immunoprecipitation and immunoblotting procedures

Confluent HUVECs were incubated for 18 h in EBM-2 containing 1% FBS. Following this, HUVECs were treated with vehicle or propranolol or PTK787 (10 μ M) or U0126 (5 μ M) for 1 h in the absence of serum and then stimulated with VEGF (50 ng/mL, 2 min). HUVECs were washed once with ice-cold PBS containing 1 mM of each NaF and Na₃VO₄ and were incubated in the same medium for 1 h at 4 °C. The cells were solubilized on ice in lysis buffer containing 1 mM of each NaF and Na₃VO₄ and the resulting lysates were processed for immunoprecipitation studies as previously described (Lamy et al., 2006a). Briefly, lysates (200 μ g protein) from each sample were incubated in lysis buffer overnight at 4 °C in the presence of 1 μ g/mL of anti-VEGFR-2 antibodies and immune complexes were collected by incubating the mixture with 25 μ L (50% suspension) of Protein A-Sepharose beads for 2 h. Bound proteins were solubilized in two-fold concentrated Laemmli sample buffer, boiled 4 min, and resolved by SDS-PAGE (7.5% gel). For analysis of ERK phosphorylation, HUVECs were stimulated with VEGF (50 ng/mL, 2 min) or norepinephrine (10 μ M, 15 min) and lysed as previously described. Lysates (20 μ g) from control and treated cells were then solubilized in Laemmli sample buffer, boiled for 4 min and resolved on 10% SDS-PAGE. Following electrophoresis, proteins were transferred onto PVDF membranes, blocked overnight at 4 °C with TBS-T containing 3% BSA and incubated 1 h at room temperature with primary antibodies. Immunoreactive bands were revealed after 1 h incubation with HRP-conjugated anti-rabbit antibodies and the signals were visualized by enhanced chemiluminescence.

2.10. cAMP assay

Intracellular cAMP assay was performed according to the manufacturer's recommendation (Cell Biolabs). In brief, cells were treated in the presence or absence of VEGF (50 ng/mL), epinephrine (100 μ M) or propranolol (25 μ M) in EBM-2 for 1 h. The cAMP level was then measured by using the cAMP ELISA Kit, a competitive enzyme immunoassay method.

2.11. Gelatin zymography

Gelatin zymography was used to assess the extent of proMMP-2 activity as previously described (Lamy et al., 2006b). Briefly, quiescent

HUVECs were serum-starved for 6 h in the presence or absence of propranolol (25 μ M) or PTK787 (10 μ M) and then stimulated with VEGF (50 ng/mL) or norepinephrine (10 μ M) for 18 h. The conditioned media were then collected and clarified by centrifugation. An aliquot (20 μ L) of the culture medium was subjected to SDS-PAGE in a gel containing 1 mg/mL gelatin. The gels were then incubated in 2.5% Triton X-100 and rinsed in nanopure distilled H₂O. The gels were further incubated at 37 °C for 18 h in 200 mM NaCl/5 mM CaCl₂/0.02% Brij-35/ 50 mM Tris-HCl buffer (pH 7.6), then stained with 0.1% Coomassie Brilliant Blue R-250, followed by destaining in 10% acetic acid/30% methanol. Gelatinolytic activity was detected as unstained bands on a blue background.

2.12. Statistical analysis

Statistical analyses were performed with Student's t-test when one group was compared with the control group. To compare two or more groups with the control group, one-way analysis of variance (ANOVA) with Dunnett's post hoc test was used. All statistical analyses were performed using GraphPad Prism software). Differences with $P < 0.05$ were considered significant.

3. Results

3.1. Propranolol inhibits growth factor-induced EC proliferation

The proliferation of vascular ECs is one of the important events during angiogenesis (Folkman, 2002). In order to investigate whether propranolol was able to inhibit EC proliferation, HUVECs were cultured in 96-well plates and incubated with propranolol for 30 h in a concentration range of 5 to 100 μ M. Since apoptosis is triggered in HUVECs cultured under serum-free condition (Zoellner et al., 1996), cell proliferation assay was performed in the presence of FBS (5%), an important source of growth factors (Gstraunthaler, 2003). As shown in Fig. 1, the growth of HUVECs was strongly inhibited by propranolol in a dose-dependent manner with a half-maximal inhibition (IC₅₀) of 21 μ M without toxicity, confirming the specific inhibitory effect of propranolol on EC proliferation.

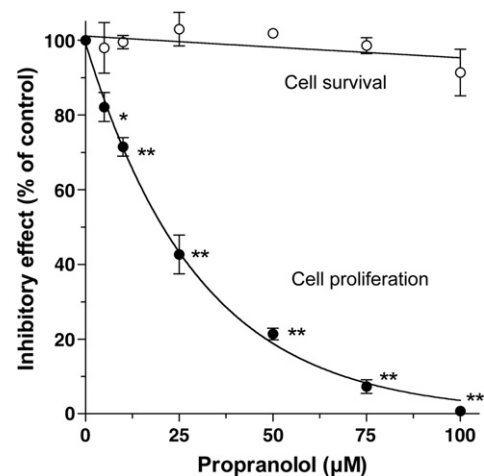


Fig. 1. Propranolol inhibits growth factor-induced proliferation without affecting HUVECs viability. Cells were incubated for 30 h in the presence of 5% FBS with various concentrations (5, 10, 25, 50, 75 or 100 μ M) of propranolol (●) and the extent of cell proliferation was measured using the WST-1 assay. Cell viability (○) was estimated by the analyzing the content of LDH in the culture medium of each well. Values are means of three independent experiments (* $p < 0.05$; ** $p < 0.01$ versus growth factors alone); bars, \pm SEM.

3.2. Propranolol induces G₁ phase arrest of ECs

To further investigate the cellular mechanism of propranolol-mediated growth inhibition, FACS analyses of DNA content in both vehicle- and propranolol-treated HUVECs were conducted under the same conditions as for cell proliferation (5% FBS). Fig. 2A shows that treatment of HUVECs with propranolol induced an accumulation of cells at the G₀/G₁ phase of the cell cycle, associated with a reduction in the S and M phases as compared to the cells treated with vehicle alone. This suggests that the growth inhibitory effect of propranolol is

due to an arrest of DNA replication, thereby inhibiting further progress in the cell cycle. Propranolol at concentrations of 10, 25, 50, and 100 μM for 20 h effectively decreased the distribution of cells in the S phase by 23.6%, 23.3%, 50.8%, and 71.6%, respectively (Fig. 2B, top panel). Moreover, treatment of HUVECs with 50 μM propranolol for various periods of time (0, 6, 18, and 30 h) was also able to block cell cycle progression from G₀/G₁ to S phase (Fig. 2B, bottom panel). After 30 h of treatment, histograms showed an increase of 45.7% of cells in G₀/G₁ and concomitant reductions of 71.7% and 56.4% of cells in S and G₂/M, respectively. These findings indicate that propranolol

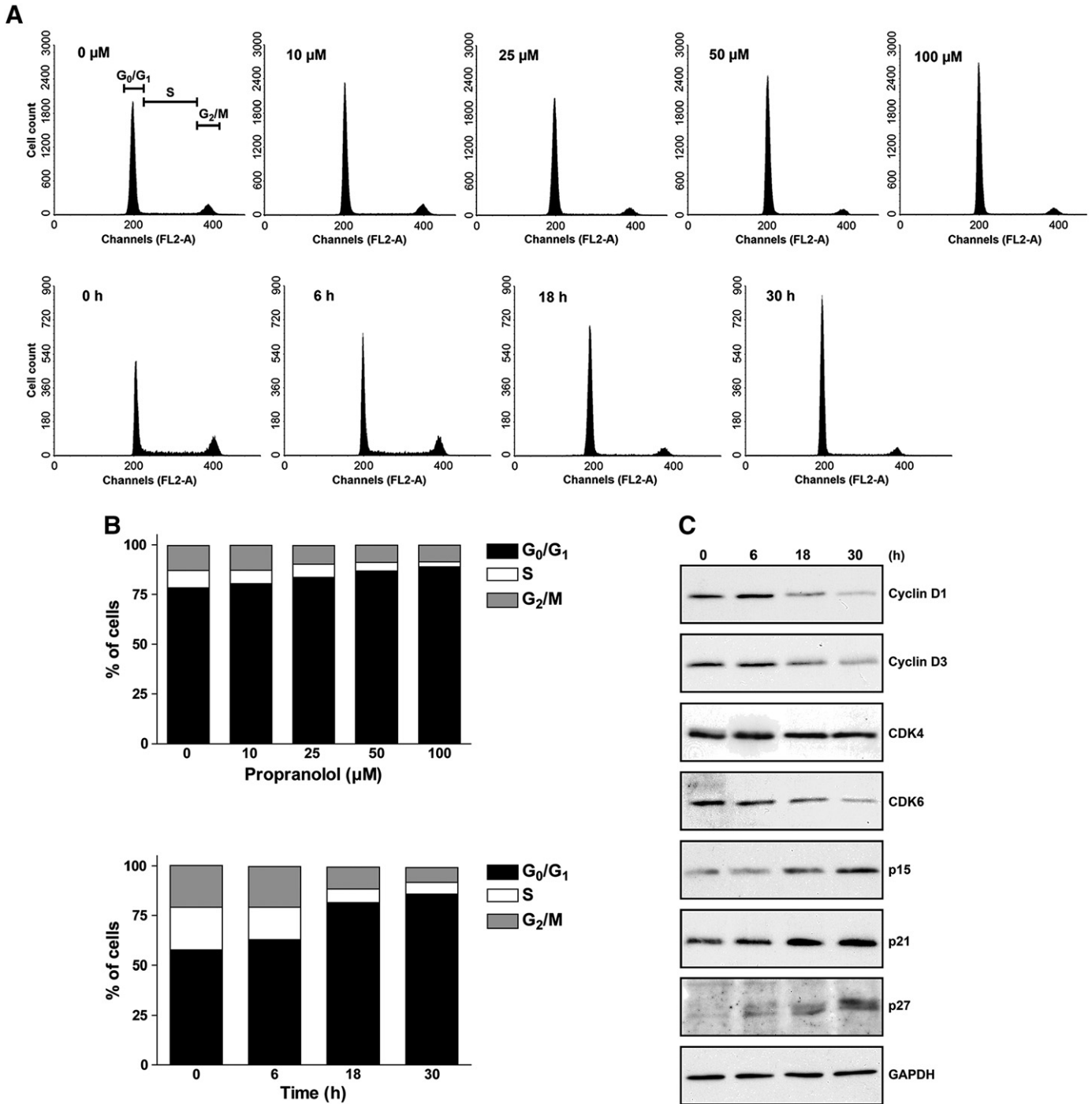


Fig. 2. Propranolol inhibits growth factor-induced cell cycle progression in HUVECs. Cells were exposed to either control medium (containing 5% FBS) or medium containing the indicated concentrations of propranolol and were treated as indicated in Materials and methods section. (A) Representatives cytometric profiles and (B) histogram distribution of cells in the G₀/G₁, S and G₂/M phases as determined by flow cytometric analysis. The individual DNA content was determined by fluorescence intensity of incorporated propidium iodide. (C) Western blot of cell-cycle regulatory proteins in HUVECs treated with 50 μM propranolol for various time periods (6, 18, and 30 h). Total GAPDH was used for normalization. Data are representative of three independent experiments.

markedly affects the transition of HUVECs from G₁ to S phase in a dose- and time-dependent manner.

3.3. Propranolol modulates different proteins involved in the regulation of G₁ phase

To characterize the mechanisms of propranolol-induced cell cycle arrest, the effects of propranolol on cell cycle regulatory molecules, which are operative in the G₁ phase and G₁/S transition, were determined. When HUVECs were treated with 50 μM propranolol for various time periods, the expressions of cyclin D1, cyclin D3, and CDK6 were significantly reduced in a time-dependent manner. However, propranolol did not induce any significant changes in the protein levels of CDK4 (Fig. 2C). Since CDK activity can also be controlled by a group of protein inhibitors (CKIs), we further examined the changes in protein levels of p15^{INK4B}, p21^{WAF1/Cip1} and p27^{Kip1} in propranolol-treated cells. As shown in Fig. 2C, the expression of these proteins was time-dependently increased in the propranolol-treated HUVECs. Taken together, these results suggest that the inhibitory effect of propranolol on cell proliferation is accompanied by modulation of cell cycle progression.

3.4. Propranolol inhibits growth factor-induced migration and tube formation of ECs

Migration of ECs represents another critical step in angiogenesis, allowing cells to disseminate from pre-existing vessels and to form new vessels. Therefore, the effect of propranolol on chemotactic motility of HUVECs was investigated. Cells were allowed to adhere to gelatin-coated Transwells and were incubated with various concentrations of propranolol. In order to approximate tumor-mediated angiogenesis conditions, EC migration was monitored in response to growth factor-enriched media isolated from U87 glioma cells, which was added to the lower chamber and served as a chemoattractant. We studied the effect of propranolol on cell migration only after growth factors stimulation since, under basal control conditions HUVEC migration is very low (Lamy et al., 2006a). As shown in Fig. 3A, propranolol potently inhibited the growth factor-induced migration of HUVECs in a dose-dependent manner. We next investigated the effect of propranolol on the morphological differentiation of ECs into capillary-like structures using *in vitro* tube formation assay on a solubilized basement membrane preparation (Matrigel) extracted from Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins and growth factors (Yang et al., 2003). As shown in Fig. 3B, propranolol abrogated the width and the length of endothelial tubular structures in a concentration-dependent manner at pharmacological dosages relevant for human beings (Masur et al., 2001) (IC₅₀ = 8 μM). Moreover, the tubulogenesis was completely abolished by propranolol at 50 μM. Interestingly, propranolol is also capable of inhibiting the tube formation of microvascular ECs, such as HMVECs (Fig. 3C) and human brain microvascular ECs (HBMECs) (Annabi et al., 2009). Overall, these results demonstrate that propranolol has the ability to block growth factor-induced angiogenesis.

3.5. Propranolol inhibits VEGF-induced tyrosine phosphorylation of VEGFR-2

VEGF has been shown to be the central positive regulator of the early growth of neovessels. Inhibition of its cell surface receptor (VEGFR-2) activity, which plays a crucial role in proliferation and migration of ECs, limits the ability of most tumors to stimulate the formation of blood vessels (Grimm et al., 2009). Therefore, we examined whether propranolol could have an effect on VEGF-induced tyrosine phosphorylation of VEGFR-2. Quiescent HUVECs were pre-incubated for 1 h in the presence of various concentrations of propranolol or PTK787 (10 μM) and then stimulated with 50 ng/mL

VEGF for 2 min. The extent of VEGFR-2 phosphorylation was assessed by immunoprecipitation of the receptor, followed by immunoblotting using monoclonal anti-Tyr(P) antibody. Under these experimental conditions, we observed that propranolol inhibited VEGF-induced tyrosine phosphorylation of VEGFR-2 in a dose-dependent manner with an IC₅₀ of 18 μM. The inhibitory effect of propranolol on VEGF receptor functions could be compared to that of PTK787, a known pharmacological inhibitor of the tyrosine kinase activity associated to VEGFR-2 (Fig. 4A).

3.6. Propranolol inhibits VEGF-induced ERK phosphorylation

Following VEGF-induced autophosphorylation of VEGFR-2, the receptor induces activation and/or phosphorylation of several substrates such as ERK-1/2 (Cross et al., 2003). Since numerous studies have shown that the ERK signaling pathway is involved in many important cellular processes of angiogenesis, including those involved in EC migration and proliferation (Katz et al., 2007), we examined the phosphorylation level of ERK-1/2 induced by VEGF. Propranolol inhibited the tyrosine phosphorylation of ERK-1/2 induced by VEGF with ~58% inhibition (Fig. 4B, left panel). Since MAPKs can also be stimulated by neurotransmitters (Seger and Krebs, 1995) and β-ARs are expressed in ECs (Ferro et al., 1999; Howell et al., 1988), we explored whether ERK/MAPK activation by the catecholamine norepinephrine could be inhibited by propranolol. As shown in Fig. 4B (right panel), propranolol inhibited the tyrosine phosphorylation of ERK-1/2 less effectively (~24% inhibition). Moreover, the treatment of HUVECs with the MEK inhibitor U0126 completely inhibited ERK activation induced by VEGF or norepinephrine. Therefore, these results argue against a nonspecific effect of propranolol on the ERK pathway, suggesting that β-ARs signaling may not be the only pathway affected by propranolol. In order to verify this assumption, we next examined the effect of propranolol on the intracellular levels of cAMP in HUVECs, since β-adrenergic stimulation enhances cAMP and affects EC functions (Tilan and Kitlinska, 2010). Incubation for 1 h with epinephrine, a cAMP elevating agent working in HUVECs (Sung et al., 1991), significantly increased cAMP levels, whereas VEGF had no effect (Fig. 4C). Thus, these data indicate that the antiangiogenic effect of propranolol is not related to a change of cellular cAMP concentration after VEGF stimulation.

3.7. Propranolol inhibits VEGF-induced proMMP-2 secretion

Matrix metalloproteinases (MMPs) secreted by ECs play a key role in the processes of matrix remodeling and ECs sprouting during angiogenesis (Schnaper et al., 1993). Recently, our laboratory reported that propranolol inhibited tubulogenesis in HBMECs through the inhibition of MMP-9 secretion (Annabi et al., 2009). While proMMP-9 is absent in basal conditions, proMMP-2 secretion can, however, be increased by VEGF in HUVECs (Lamy et al., 2006b). Thus, we assessed by gelatin zymography of the conditioned media of serum-starved HUVECs whether propranolol could have an effect on proMMP-2 secretion. After 6 h of starvation in the presence of propranolol, cells were then stimulated with VEGF or norepinephrine. A subsequent 24 h treatment shows that propranolol effectively downregulated VEGF- or norepinephrine-induced proMMP-2 levels by ~98.3% and ~53.1%, respectively (Fig. 5). Interestingly, the inhibitory effect of propranolol on VEGF-induced proMMP-2 extracellular levels was similar to that achieved by the inhibitor of the VEGFR-2, PTK787 (~96.6% inhibition). These results suggest that the effect of propranolol toward MMP secretion is indeed regulated through VEGFR-2 pathway.

4. Discussion

β-Adrenoceptor, a classical G-protein-coupled receptor (GPCR), originally identified as an important regulator of cardiac contractility and smooth muscle relaxation, is now emerging as a multifunctional

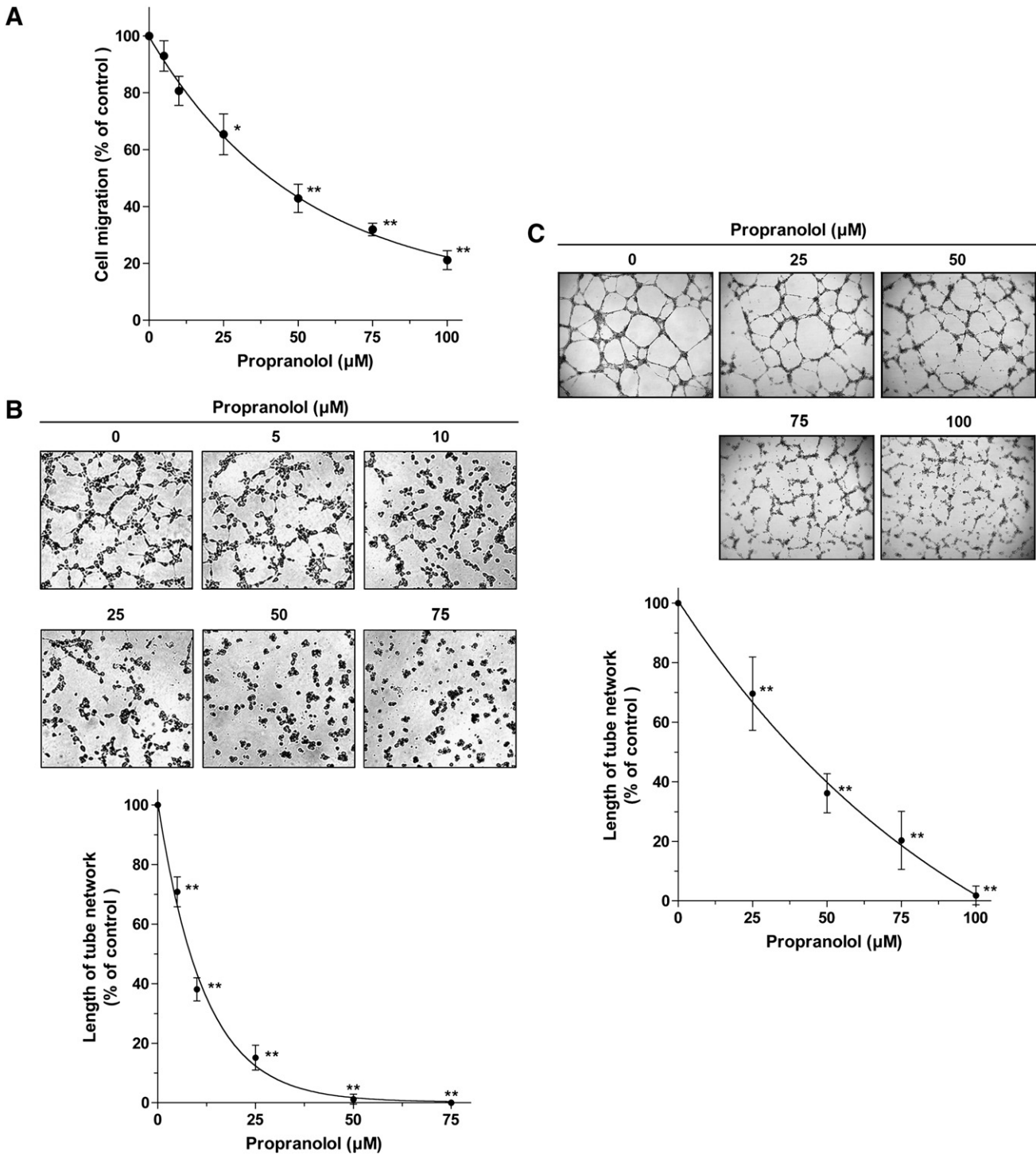


Fig. 3. Propranolol inhibits growth factor-induced migration and tube formation of ECs. (A) HUVECs were pretreated for 2 h with various concentrations (5, 10, 25, 50, 75 or 100 μM) of propranolol before stimulation with conditioned media from U87. After 4 h incubation with growth factors, migration was quantified by counting the cells that crossed the membrane to the lower side of the filter with optical microscopy at ×50 magnification. The number of cells which migrated was compared to that observed with growth factor-treated cells. Values are means of three independent experiments (**p*<0.05; ***p*<0.01 versus growth factors alone); bars, ± SEM. (B) HUVECs and (C) HMVECs were cultured on Matrigel-coated 96-well plates at a density of 2 × 10⁴ cells/well in serum-free medium. After adhesion of cells for 30 min, propranolol was added at the indicated concentrations for 6 h (HUVECs) and 18 h (HMVECs). The length of the tube network was quantitated using Northern Eclipse software. Values are means of two independent experiments (***p*<0.01 versus control alone); bars, ± SD.

cell surface catecholamine sensor that regulates a repertoire of cellular processes essential for angiogenesis such as cell proliferation, migration, and apoptosis (Schuller, 2007). However, the effects of β-blockers on angiogenesis are currently unknown. Recent evidence suggests that propranolol, a non-selective β-adrenergic antagonist,

can be used to treat severe hemangiomas, the most common tumors of infancy (Leaute-Labreze et al., 2008). While hemangiomas are composed of a heterogeneous population of cells, it has been hypothesized that their development is consequent to an abnormal EC proliferation causing a dysregulation of angiogenesis (Folkman,

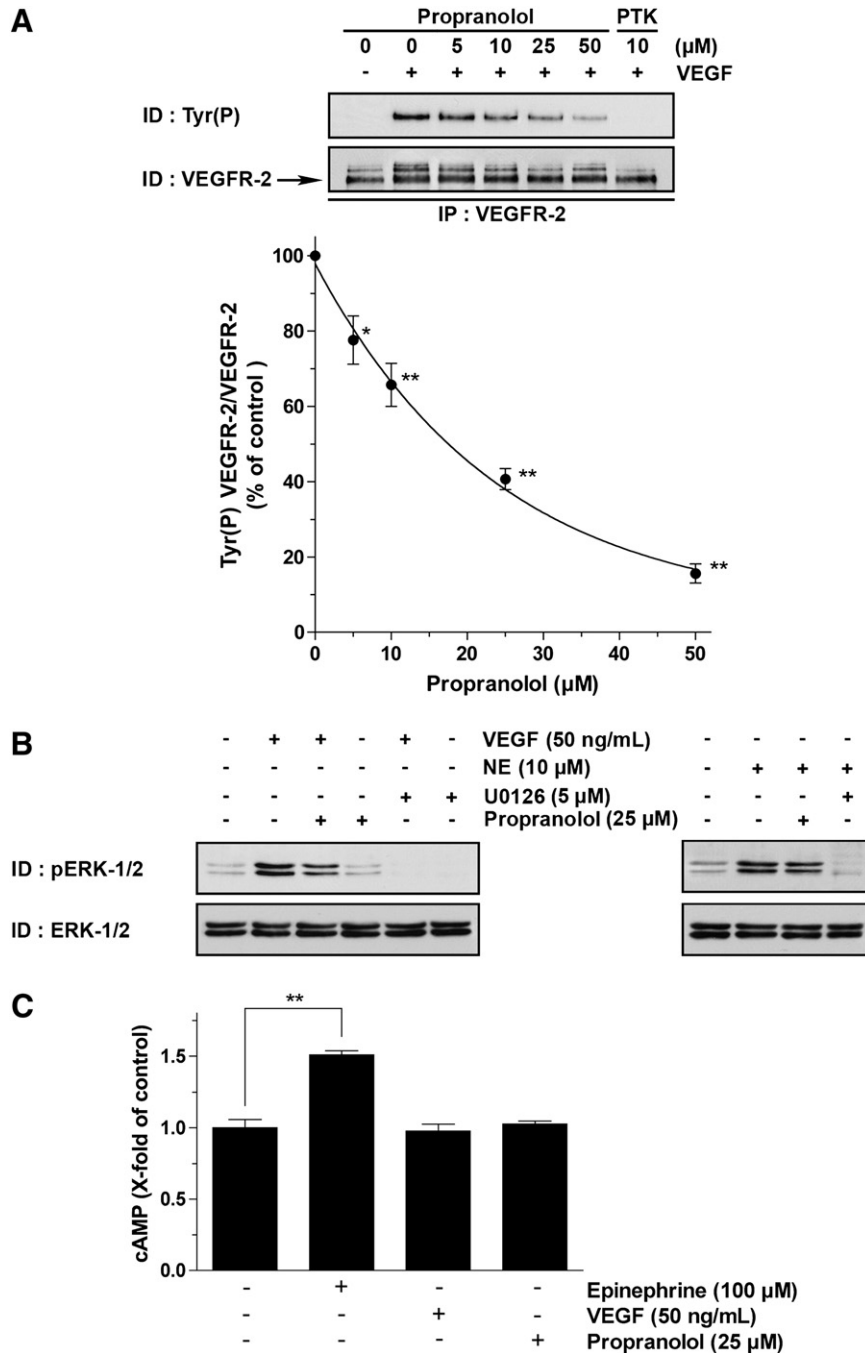


Fig. 4. Propranolol inhibits VEGFR-2 and ERK-1/2 phosphorylation. Quiescent HUVECs were incubated in 1% FBS for 18 h. The medium was then replaced with serum-free medium in the presence or not of PTK787 or U0126 or propranolol at the indicated concentrations for 1 h before adding VEGF or norepinephrine (NE). (A) Cells were lysed and the extent of VEGFR-2 tyrosine phosphorylation was monitored by immunoprecipitation with anti-VEGFR-2 and immunoblotting with anti-Tyr(P) monoclonal antibody (PY99). Results were analyzed by densitometry and values are means of three independent experiments (* $p < 0.05$; ** $p < 0.01$ versus VEGF alone); bars, \pm SEM. (B) Equal amounts of protein were separated by SDS-PAGE electrophoresis and the phosphorylated forms of ERK-1/2 (top panel) and the effects of the treatments on the amount of ERK-1/2 (bottom panel) were visualized by immunoblotting using specific antibodies. Data are representative of three independent experiments. (C) Intracellular levels of cAMP were measured in HUVECs treated in the presence or absence of VEGF, epinephrine or propranolol for 1 h as described in Materials and methods. Values are means of two independent experiments (** $p < 0.01$ versus control alone); bars, \pm SD.

1995b) with a high expression of both VEGF and bFGF during the proliferative phase (Takahashi et al., 1994). Although propranolol appears to have impressive beneficial effects on severe hemangiomas of infancy, research to further assess propranolol's effect on hemangiomas is necessary to understand its potential antiangiogenic activity.

Since there are a number of similarities between normal blood vessels and the structures found in these infantile tumors (Ritter et al.,

2007), we investigated the effect of propranolol on angiogenesis using a well-established primary EC model (Bouis et al., 2001). Here, we show that propranolol inhibits the proliferation of HUVECs, a critical step in angiogenesis (Risau, 1997). Since propranolol does not have a cytotoxic effect up to a concentration of 100 μM , the antiproliferative effect may not be due to apoptosis or necrosis of ECs, but rather to the inhibition of cell cycle progression induced by growth factors. Propranolol arrested cells at the G_0/G_1 phase of the cell cycle, in part through the inhibition of

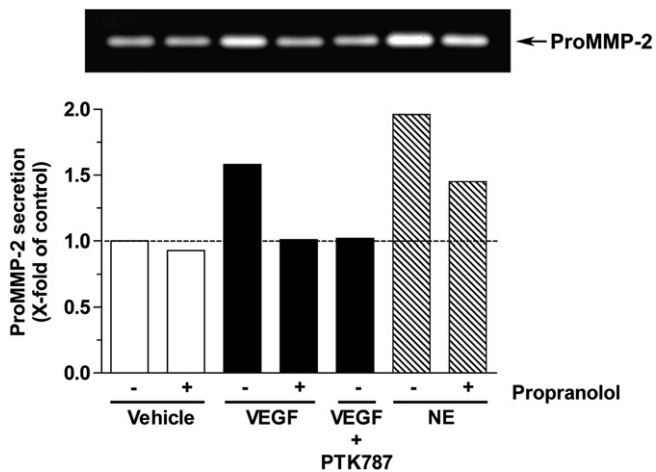


Fig. 5. Propranolol inhibits proMMP-2 secretion. HUVECs were serum-starved and conditioned media isolated from control or VEGF- or norepinephrine (NE)-treated cells in the presence or not of 25 μ M propranolol or 5 μ M PTK787. Zymography (20 μ L/well) was performed by SDS-PAGE in gels containing gelatin, as described in the Methods section. Quantification of the extent of gelatin hydrolysis was performed by densitometry. Data are representative of two independent experiments.

specialized molecular players involved in driving cells through G_1 into S phase. The inhibition of cell proliferation was also correlated with reduced chemotactic motility, as well as reduced EC ability to differentiate into capillary-like structures, all events critical for new vessel sprouting (Liekens et al., 2001; Risau, 1997). Interestingly, it has been reported that cyclin D1 also has a role in mediating cell migration (Li et al., 2006). Moreover, overexpression of p27^{Kip1} in HUVECs was reported to inhibit cell proliferation, cell migration, as well as tubulogenesis (Goukassian et al., 2001).

The ability of growth factors to stimulate EC proliferation may be key to their roles as promoters of angiogenic responses in pathological settings (Castellon et al., 2002). Indeed, VEGF, the most critical angiogenic promoter (Pandya et al., 2006), initiates EC proliferation, elongation and reorientation, transforming their morphology to the highly ordered, elongated phenotype of cells lining the inner surface of blood vessels (Zhao et al., 2004). Interestingly, hemangioma-derived ECs are characterized by constitutively active VEGFR-2 signaling suggesting that local administration of antibodies that neutralize VEGF or inhibitors of VEGFR-2 tyrosine kinase activity could be effective in rapidly treating growing hemangiomas that do not respond to standard therapy (Jinnin et al., 2008). It is, therefore, possible that propranolol also affects VEGF-stimulated EC functions relevant to the angiogenic process. Consequently, we investigated whether the mechanism of antiangiogenic effect of propranolol involves the inhibition of receptor tyrosine kinase (RTK) activity of VEGFR-2. We observed that propranolol effectively inhibits VEGF-dependent tyrosine phosphorylation of the receptor in a dose-dependent manner and that this inhibitory effect is associated with an impairment of downstream signaling events triggered by VEGFR-2, such as phosphorylation of the p42^{MAPK} and p44^{MAPK} forms (ERK-1/2). To the best of our knowledge, this is the first study to highlight the inhibitory effect of propranolol on this important RTK.

In an attempt to assess the contribution of the β -ARs expressed in HUVECs, we studied the effect of the β -blocker propranolol on the phosphorylation level of ERK-1/2 induced by norepinephrine. We observed a complete inhibition of ERK activation induced by U0126, whereas propranolol was less potent, in agreement with previous reports that suggest that the stimulatory effect of norepinephrine on ERK could rather be mediated by the α_1 -adrenoceptor (deBlois et al., 1996; Seya et al., 2006). Moreover, propranolol nearly abolished MMP-2 secretion induced by VEGF and, to a lesser extent, by norepinephrine

suggesting overall that the modulation of angiogenesis by propranolol involved other pathways.

There is evidence that the expression of VEGF is also controlled by adrenergic stimulation, as demonstrated in different *in vitro* and *in vivo* models of VEGF (Thaker et al., 2006; Yang et al., 2006). The activation of β -ARs present on tumor cells increases in cAMP levels, which results in the activation of protein kinase A and Src, and leads to an increase in synthesis and release of angiogenic factors (Thaker et al., 2006; Yang et al., 2006, 2009). Since β -adrenergic stimulation increases cAMP, we examined whether the antiangiogenic effect of propranolol depends on modulating the cellular cAMP concentration in HUVECs. The results show that VEGF cannot induce cAMP in short time incubation conditions indicating again that propranolol could exert its antiangiogenic effect on a specific pathway involving VEGFR-2 signaling. A study has shown that the β -blocker Carvediol also inhibited RTK autophosphorylation of the PDGFR- β , in human cardiac fibroblasts (Lotze et al., 2002). Although the exact mechanisms underlying the inhibitory effect of propranolol on VEGFR-2 remain to be determined, it is possible that, similarly to PTK787 (Liu et al., 2009), propranolol could be a competitive inhibitor (in regard to ATP-binding sites) of the tyrosine kinase activity of VEGFR-2, thereby inhibiting its tyrosine phosphorylation and the signaling cascade triggered by VEGF.

5. Conclusion

Our investigations have revealed novel anti-EC functions of propranolol based on multiple molecular targeting of critical steps in the angiogenic cascade. Our study provides the first evidence that the inhibitory effect of propranolol on angiogenesis could not only involve inhibitory effects on β -ARs signaling but also inhibition of the VEGFR-2 pathway. Although further studies are needed to better understand the different mechanisms of propranolol-induced inhibition of angiogenesis, these results provide further justification for the investigation of the use of β -blockers for the treatment of hemangiomas and other angiogenesis-dependent human diseases.

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