

Forskolin increases angiogenesis through the coordinated cross-talk of PKA-dependent VEGF expression and Epac-mediated PI3K/Akt/eNOS signaling

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ABSTRACT

Forskolin, a potent activator of adenylyl cyclases, has been implicated in modulating angiogenesis, but the underlying mechanism has not been clearly elucidated. We investigated the signal mechanism by which forskolin regulates angiogenesis. Forskolin stimulated angiogenesis of human endothelial cells and *in vivo* neovascularization, which was accompanied by phosphorylation of CREB, ERK, Akt, and endothelial nitric oxide synthase (eNOS) as well as NO production and VEGF expression. Forskolin-induced CREB phosphorylation, VEGF promoter activity, and VEGF expression were blocked by the PKA inhibitor PKI. Moreover, phosphorylation of ERK by forskolin was inhibited by the MEK inhibitor PD98059, but not PKI. The forskolin-induced Akt/eNOS/NO pathway was completely inhibited by the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, but not significantly suppressed by PKI. These inhibitors and a NOS inhibitor partially inhibited forskolin-induced angiogenesis. The exchange protein directly activated by cAMP (Epac) activator, 8CPT-2Me-cAMP, promoted the Akt/eNOS/NO pathway and ERK phosphorylation, but did not induce CREB phosphorylation and VEGF expression. The angiogenic effect of the Epac activator was diminished by the inhibition of PI3K and MEK, but not by the PKA inhibitor. Small interfering RNA-mediated knockdown of Epac1 suppressed forskolin-induced angiogenesis and phosphorylation of ERK, Akt, and eNOS, but not CREB phosphorylation and VEGF expression. These results suggest that forskolin stimulates angiogenesis through coordinated cross-talk between two distinct pathways, PKA-dependent VEGF expression and Epac-dependent ERK activation and PI3K/Akt/eNOS/NO signaling.

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1. Introduction

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is a fundamental step in several physiological events including

Abbreviations: eNOS, endothelial nitric oxide synthase; NO, nitric oxide; PI3K, phosphatidylinositol 3-kinase; VEGF, vascular endothelial growth factor; Epac, exchange protein directly activated by cAMP; HUVECs, human umbilical endothelial cells; PKA, cAMP-dependent protein kinase A; PGE₂, prostaglandin E₂; DAF-FM/DA, 4-amino-5-methylamino-2'-7'-difluorofluorescein diacetate; 8CPT-2Me-cAMP, 8-(*p*-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate; NMA, N-monomethyl-L-arginine; DB-cAMP, dibutyryl-cAMP; DAF-FM/DA, 4-amino-5-methylamino-2'-7'-difluorofluorescein diacetate; siRNA, small interfering RNA.

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embryonic development, the female reproductive cycle, placentation, and wound repair. It also plays an important role in pathological conditions, such as tumor growth and metastasis, rheumatoid arthritis, and diabetic retinopathy. In addition, the elevation of angiogenesis is an important homeostatic process contributing to the ischemic tissues of myocardial infarction and stroke. Angiogenesis is a complex multistep process which involves the stimulation of endothelial growth, degradation of extracellular matrix proteins, migration of endothelial cells, and morphological differentiation of endothelial cells to form tubes [1,2]. This process is tightly controlled by a wide variety of biologically active substances, such as growth factors, cytokines, lipid metabolites, and cryptic fragments of hemostatic proteins [3].

Pro-angiogenic regulators are classified into two groups: the first group is comprised of direct inducers such as the vascular endothelial growth factor (VEGF) and the basic fibroblast growth factor which can promote the angiogenic process via activation of angiogenic signal pathways. The second group is indirect inducers including TNF- α , transforming growth factor- β , and interleukin-1 β that act on

endothelial cells to induce the production of direct angiogenic factors from accessory cells such as immune cells and tumor cells [3]. Direct angiogenic factors improve vascular remodeling through the activation of several intracellular signaling pathways including MAPKs, phosphoinositide-3 kinase (PI3K)/Akt, and FAK/Paxillin. In addition, nitric oxide (NO), synthesized by endothelial nitric oxide synthase (eNOS), has been shown to play an important role in angiogenic factor-induced angiogenesis by elevating the intracellular level of cGMP through the activation of soluble guanylyl cyclase [4–6].

Of the various pro-angiogenic inducers, adenylyl cyclase-activating biomolecules, such as prostaglandin E₂ (PGE₂) [7], thyrotropin [8], parathyroid hormone-related peptide [9], and norepinephrine [10], have been shown to promote the angiogenic process via the activation of cAMP-dependent protein kinase A (PKA), a major cellular receptor for cAMP [11,12], through the elevation of intracellular cAMP levels. cAMP, intimately identified as a metabolic regulator, has been shown to play a role of an intracellular second messenger in a wide variety of pathophysiological processes via the activation of PKA and a newly recognized family of cAMP-binding proteins designated as Epac (exchange protein directly activated by cAMP). It suggests that these two distinct signal pathways are involved in cAMP-dependent endothelial cell survival, enhancement of endothelial cell barrier function [13], and angiogenesis [14,15]. Moreover, membrane permeable cAMP-analogues have also been shown to promote angiogenesis via the elevation of VEGF expression and the activation of the PI3K/Akt and eNOS/NO pathways [7,16,17].

Forskolin, a potent and unique activator of adenylyl cyclase, enhanced various endothelial events, including angiogenesis by elevating the intracellular cAMP level [18,19]. There is scant information on the functional role of PKA and/or Epac and coordinated cross-talk between them in forskolin-induced angiogenesis and its signaling mechanism. To characterize the contribution of PKA and Epac in angiogenesis, we have comprehensively dissected the molecular mechanism and signaling pathway by which forskolin regulates the angiogenic process in cultured human umbilical endothelial cells (HUVECs). Herein, we demonstrated that forskolin plays a significant role in facilitating angiogenesis both via PKA-mediated VEGF expression and Epac-dependent ERK, Akt, and eNOS activation.

2. Materials and methods

2.1. Materials

The following agents were purchased: LY294002, PD98059, N-monomethyl-L-arginine (NMA), dibutyryl-cAMP (DB-cAMP), and myristoylated protein kinase A inhibitor amide 14–22 (PKI) from Calbiochem (San Diego, CA); growth factor-reduced Matrigel from BD biosciences (Franklin Lakes, NJ); M199 medium, penicillin, and streptomycin from Invitrogen life technologies (Carlsbad, CA); antibodies for phospho-ERK^(Thr202/Tyr204), ERK, phospho-Akt^(Ser473), Akt, phospho-eNOS^(Ser1177), and eNOS from New England Biolabs (Beverly, MA); VEGF from Upstate Biotechnology (Lake Placid, NY); M199, heparin, and Trizol were purchased from Invitrogen Life Technologies (Carlsbad, CA); 4-amino-5-methylamino-2'-7'-difluorofluorescein diacetate (DAF-FM/DA) from Molecular Probes (Eugene, OR); 8-(p-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8CPT-2Me-cAMP) from BioLog Life Sci. Institute (Bremen, Germany). All other reagents were purchased from Sigma (St. Louis, MO), unless indicated otherwise.

2.2. Cell culture and proliferation

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment as previously described [7] and used in passages 3–6. The cells were grown in M199 medium supplemented with 20% fetal bovine serum

(FBS), 100 units/ml penicillin, 100 µg/ml streptomycin, 3 ng/ml bFGF, and 5 units/ml heparin at 37 °C under 5% CO₂/95% air. HUVEC proliferation was determined by DNA synthesis [6]. Briefly, HUVECs were seeded at a density of 2.5×10^4 cells/well in gelatin-coated 24-well plates. Cells were incubated in growth media and allowed to attach for 24 h. Cells were washed twice with M199 and incubated for 6 h with M199 containing 1% FBS. Cells were stimulated with forskolin (10 µM) for 30 h, followed by the addition of 1 µCi/ml [³H]thymidine for 6 h. High molecular mass [³H]-labeled radioactivity was precipitated using 5% trichloroacetic acid at 4 °C for 30 min. After washing twice with ice-cold H₂O, [³H]-labeled radioactivity was solubilized in 0.2 N NaOH containing 0.1% sodium dodecyl sulfate and determined by a liquid scintillation counter.

2.3. Cell migration assay

Migration assays were performed as previously described [6]. Briefly, the chemotactic motility of HUVECs was assayed using Transwell (Costar, Corning, NY). The lower surface of the filter was coated with 10 µg of gelatin. Fresh M199 media (1% FBS) containing forskolin (10 µM) were placed in the lower wells. HUVECs were trypsinized and suspended at a final concentration of 1×10^6 cells/ml in M199 containing 1% FBS. Each inhibitor was incubated with cells for 30 min at room temperature before seeding. One hundred µl of the cell suspension was loaded into each of the upper wells. The chamber was incubated at 37 °C for 4 h. Cells were fixed and stained with hematoxylin and eosin. Non-migrating cells on the upper surface of the filter were removed by wiping with a cotton swab, and chemotaxis was observed using an inverted phase contrast microscope. Images were captured with a video graphic system. Chemotaxis was quantified by counting cells that migrated to the lower side of the filter at low-power fields ($\times 100$). All fields were counted for each assay. Each experiment was repeated 3 times.

2.4. Tube formation assay

The formation of vascular-like structures by HUVECs on growth factor-reduced Matrigel was performed as previously described [20]. Twenty-four well culture plates were coated with Matrigel according to the manufacturer's instructions. HUVECs were incubated in M199 containing 1% FBS for 6 h, plated onto a layer of Matrigel at a density of 2.5×10^5 cells/well, followed by the addition of forskolin (10 µM) with or without each inhibitor. Matrigel cultures were incubated at 37 °C for 20 to 26 h. Tube formation was observed using an inverted phase contrast microscope and images were captured with a video graphic system. The degree of tube formation was quantified by measuring the length of tubes in 5 randomly chosen low-power fields ($\times 100$) from each well using the Image-Pro Plus v4.5 (Media Cybernetics).

2.5. Western blotting

HUVECs were serum-starved in M199 (1% FBS) for 6 h, prior to addition of forskolin or inhibitors. Cells were scraped off the plates and lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet-P40, 0.5% deoxycholic acid, 0.1% sodium dodecyl sulfate). Cell lysates (50 µg protein) were electrophoresed on SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. Membranes were incubated with antibodies against ERK, phospho-ERK^(Thr202/Tyr204), eNOS, phospho-eNOS^(Ser1177), Akt, and phospho-Akt^(Ser473). After incubation, the corresponding secondary antibody signals were detected by enhanced chemiluminescence reagents.

2.6. Intracellular NO measurement

The production of NO in HUVECs was measured using the DAF-FM/DA detection system (Invitrogen life technologies, Carlsbad, CA)

according to the manufacturer's instructions [6]. In brief, the cells were pretreated with each inhibitor for 30 min before exposure to forskolin (10 μ M) for 30 min. The cells were incubated in DAF-FM/DA (5 μ M) for 30 min at 37 °C. The cells were then washed in flash medium and the fluorescence images were captured from at least 10 randomly selected cells per dish using a confocal laser microscope. The relative levels of intracellular NO were determined from the fluorescence intensity of DAF-FM.

2.7. *In vivo* angiogenesis assay

The *in vivo* angiogenic activity of forskolin was determined by CAM assay and intravital fluorescence microscopy [6]. For chorioallantoic membrane (CAM) assay, fertilized chick embryos were pre-incubated for 10 days at 38 °C with 70% humidity. A hole was drilled over the air sac at the end of the eggs, and a 1 × 1-cm window in the shell was made to expose the CAM. Thermanox discs were sterilized and loaded with forskolin (2 nmol/2 μ l) or VEGF (20 ng/2 μ l). After air-drying under laminar flow, the discs were applied to the CAM surface of 10-day-old chick embryos. The windows were sealed with clear tape, and the eggs were incubated for 72 h. Intrapos (Green-Cross) was injected under the upper CAM to increase contrast between vessels and background. Capillary formation was inspected using a light microscope. For intravital microscopic analysis, wild type and eNOS knockout mice (male, 6–8 weeks old, Orient, Sungnam, Korea) were implanted with titanium windows (19 mm outer diameter, 14 mm inner diameter, and 0.7 mm thick) between the skin and abdominal wall following anesthetization with the inhalation of isoflurane 1.5% and a mixture of O₂/N₂O. Then, Matrigel (100 μ l, BD Biosciences, Franklin Lakes, NJ) containing forskolin (10 nmol) or VEGF (100 ng) in the presence or absence of NMA (5 mM) was put into the window inner space and a circular glass coverslip was placed on top and fixed by a snap ring. After 4 days, animals were anesthetized and received an intravenous injection of 50 μ l of 25 mg/ml fluorescein isothiocyanate-labeled dextran (MW 250,000) via the tail vein. Fluorescence images were recorded in five random locations of each window by Zeiss Axiovert 200 M microscope using a 100-W mercury lamp and filter set for blue light (excitation, 440–475 nm; emission 530–550 nm) and digitized for subsequent off-line analysis using the MetaMorph program (Universal Imaging Corp., Downingtown, PA). The relative angiogenic activity was scored from 0 (least positive) to 5 (most positive).

2.8. *Epac1*-specific small interfering RNA (siRNA) design and transfection

The siRNAs against *Epac1* were designed using two independent selection programs from Dharmacon and Ambion. The selected sequences were 5'-AAGGAGCAGAAGAATGTCAAT-3' for *EPAC1* against the catalytic domain on *Epac1*. The scramble sequences were 5'-CAGTCGCGTTTGC GACTGGTT-3' and were not found to be present in mammalian cells by BLAST search at NCBI. *Epac1*-specific siRNA and scramble siRNA were synthesized with Ambion silencer siRNA construction kit. HUVECs were transfected with 40 nM scramble and *Epac1*-specific siRNAs using a microporator according to the manufacturer's protocol (Digital Bio, Seoul, Korea) and cultured in complete media without antibiotics for 12 h. Cells were further cultured in complete media for 36 h, and the level of *Epac1* protein was determined by Western blot analysis.

2.9. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from HUVECs using TRIzol reagent. Five μ g of total RNA was converted to cDNA by incubation with 200 units of reverse transcriptase and 500 ng of oligo(dT) primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, and 1 mM dNTPs at 42 °C for 1 h. The levels of VEGF were determined from

cDNA by PCR. The sequence of primers and the conditions of PCR were the same as previously described [6].

2.10. Promoter activity assay

HUVECs were co-transfected with the luciferase reporter construct containing 2.7 kb of the 5'-flanking region of the human VEGF gene (gift from M. Shong, Chungnam National University School of Medicine, Korea) and plasmid cytomegalovirus- β -galactosidase (PCMV β -gal; Stratagene) using the lipofectamine method. After transfection, the cells were treated with 10 μ M forskolin in the presence or absence of several kinase inhibitors for 12 h, and luciferase assay was performed using a luciferase assay kit (Promega Corp., Madison, WI). The transfection efficiency was normalized by the value of co-transfected β -galactosidase activity.

2.11. Statistical analysis

The data are presented as the mean \pm standard deviation (S.D.) of at least three separate experiments in triplicate. Comparisons between two groups were analyzed using the Student's *t*-test, and significance was established at a *p* value < 0.05.

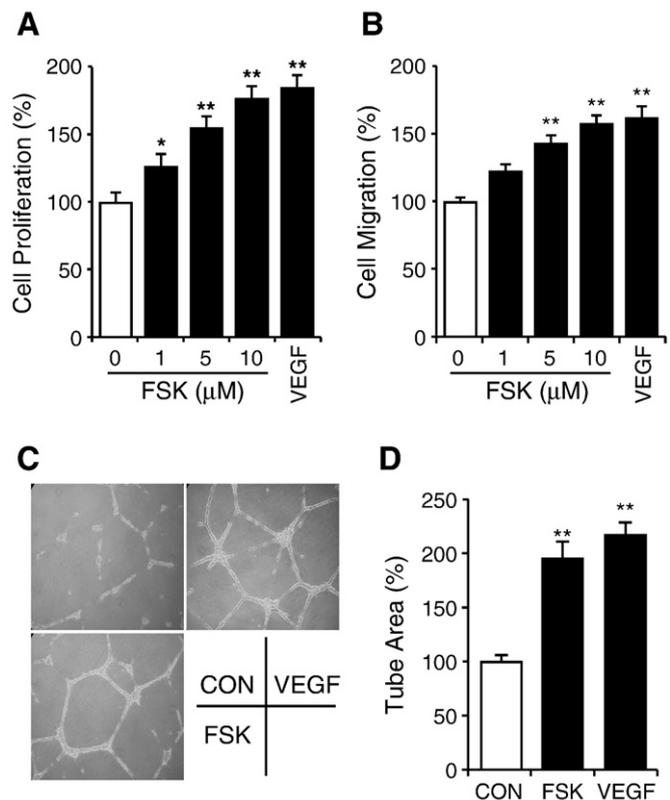


Fig. 1. Forskolin stimulates *in vitro* angiogenesis of HUVECs. (A) HUVECs were plated onto 24-well plates with the indicated concentrations of forskolin (FSK) or 10 ng/ml of VEGF for 30 h, followed by the addition of 1 μ Ci/ml [³H]thymidine for 6 h. Cell proliferation was determined by the [³H]thymidine incorporation assay. (B) HUVECs were treated with the indicated concentrations of forskolin (FSK) or VEGF in Transwell plates for 4 h. Chemotactic migration was quantified by counting the cells that migrated to the lower side of the filter with optical microscopy. (C) HUVECs were cultured on a layer of Matrigel with or without 10 μ M forskolin or 10 ng/ml VEGF for 24 h. Tube formation was observed using an inverted phase contrast microscope with a video graphic system. (D) The area covered by the tube network was quantified using Image-Pro Plus software. All quantitative data are the mean \pm SD (*n* = 4). **P* < 0.05 and ***P* < 0.01 versus control.

3. Results

3.1. Induction of endothelial cell angiogenesis by forskolin

Angiogenesis requires the three essential processes of proliferation, migration, and tube-like structure formation of endothelial cells [3]. To determine whether forskolin induces angiogenesis, the ability of forskolin as an angiogenic stimulus was assessed in *in vitro* angiogenesis models. We first examined whether forskolin increased endothelial cell proliferation. When treated with various concentrations of forskolin for 36 h, HUVEC proliferation was increased in a dose-dependent manner (Fig. 1A). The effect of forskolin on DNA synthesis in HUVECs was about 1.6-fold at 10 μ M, and this effect was comparable with that of the well-known angiogenic factor VEGF at 10 ng/ml. We next determined the effect of forskolin on the chemotactic migration of HUVECs by employing a modified Boyden chamber assay. Forskolin stimulated endothelial cell migration in a dose-dependent manner (Fig. 1B). Following forskolin (10 μ M) treatment, migration activity was about 60% higher compared with the control, and this effect was also comparable with that of VEGF (10 ng/ml) (Fig. 1B). We next examined the effect of forskolin on the morphological differentiation of HUVECs using two-dimensional Matrigel. Treatment with 10 μ M forskolin led to the formation of elongated and robust tube-like structures, which were organized by much larger number of cells compared with the control (Fig. 1C). By measuring the area covered by the tube network using an image analysis program, forskolin stimulated tube formation by about 2-fold over the control, and this effect was similar to that of 10 ng/ml VEGF (Fig. 1D). These results indicate that forskolin possesses angiogenic activity in an *in vitro* HUVEC culture system.

3.2. Forskolin induces angiogenesis *in vivo*

To determine whether forskolin is capable of promoting angiogenesis *in vivo*, experiments were performed on CAMs using Thermanox discs. After 72 h of contact with the CAM, the disc containing 20 μ g/ml

forskolin revealed significant induction of new blood vessel formation compared with the untreated control (Fig. 2A). The relative angiogenic activity of forskolin resulted in a 3.8-fold increase in the number of positive discs/total number of eggs compared with that of PBS alone (Fig. 2B). The *in vivo* angiogenic activity of forskolin was further evaluated in an established animal model by intravital microscopy. When Matrigel containing forskolin (10 nmoles) was administered into the abdominal subcutaneous area, the vascular density was significantly increased to about 3 fold compared with control, and this effect was relatively comparable with that of 100 ng VEGF (Fig. 2C and D). These results indicate that forskolin possesses potent angiogenic activity *in vivo*.

3.3. Forskolin-induced angiogenesis requires the activation of ERKs and Akt

It has been shown that activation of ERK and Akt is an important signaling event for angiogenesis [6,21]. We determined whether forskolin regulates the phosphorylation-dependent activation of ERK and Akt. Western blot analyses revealed that treatment of HUVECs with forskolin increased phosphorylation of ERK and Akt in a time-dependent manner (Fig. 3A). ERK phosphorylation was apparent 2 min after forskolin treatment, with maximum activation at 10 min, which was an earlier event than maximum phosphorylation of Akt at 20 min. Forskolin-induced activation of ERK was strongly inhibited by co-treatment with the MEK1/2 inhibitor PD98059, but not with the PKA inhibitor PKI and the PI3K inhibitor LY294002 (Fig. 3B). Phosphorylation of Akt by forskolin was inhibited by treatment with LY294002, but not with PD98059, and this phosphorylation was partially reduced by the PKA inhibitor PKI (Fig. 3B). We next tested the effects of these chemical inhibitors on forskolin-induced angiogenesis of endothelial cells. When co-treated with PKI, LY294002, and PD98059, forskolin-induced angiogenic properties, such as proliferation, migration, and tube-like structure formation of HUVEC, were significantly inhibited (Fig. 3C–E). Of them, LY294002 showed the highest inhibitory effect on forskolin-induced angiogenesis. These

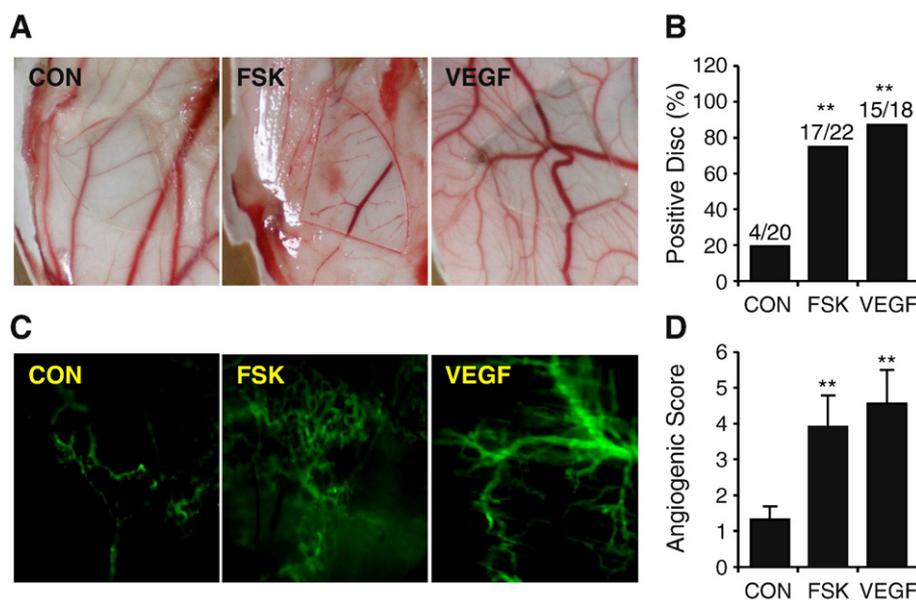


Fig. 2. Forskolin promotes *in vivo* angiogenesis. (A) Forskolin (20 μ M/2 μ l) or VEGF (20 ng/2 μ l) was loaded onto the CAMs of day 10 chick embryos. After 72 h incubation, disks and surrounding CAMs were photographed. (B) The relative angiogenic activity was determined by counting the number of positive discs/total number of eggs. (C) Matrigel (100 μ l) containing 10 nmol forskolin (10 nmol) or VEGF (200 ng) was put into the abdominal windows of mice. After 4 days, animals were anesthetized and received an intravenous injection with FITC-labeled dextran via tail vein. Fluorescence images were recorded in five random locations of each window by a Zeiss Axiovert 200 M microscope using a mercury lamp and filter set for blue light. (D) Angiogenic activity was digitized for subsequent off-line analysis using the MetaMorph program. The relative angiogenic activity was scored from 0 (least positive) to 5 (most positive). Data shown are the mean \pm SD ($n \geq 7$). ** $P < 0.01$ versus control.

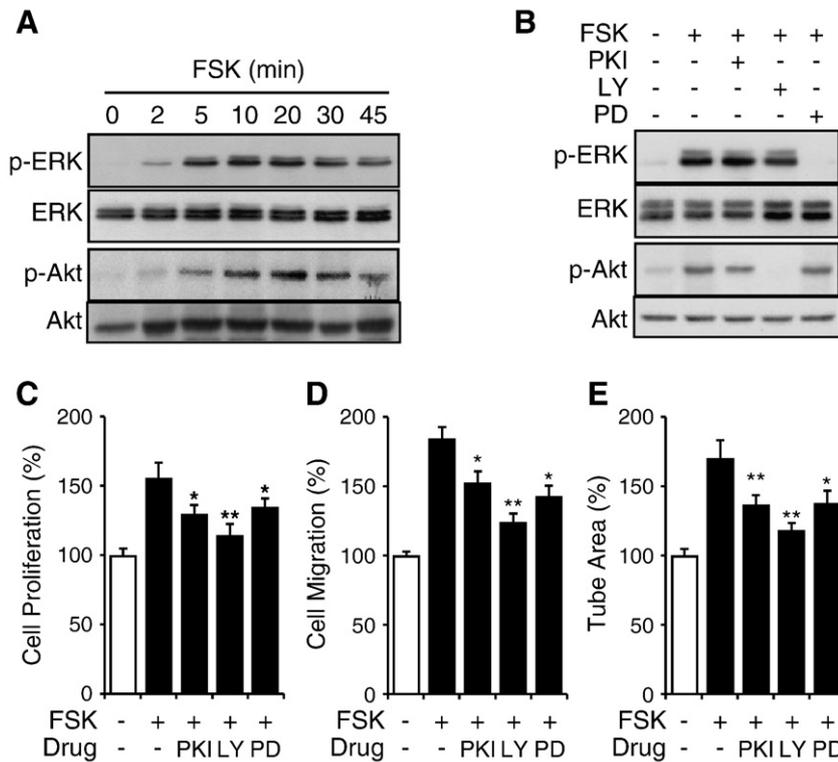


Fig. 3. Involvement of both ERK and PI3K/Akt in forskolin-mediated angiogenesis. (A) HUVECs were incubated with forskolin (10 μ M) for the indicated times. Phosphorylated ERK and Akt were determined by Western blot analyses using a specific antibody for each protein. (B) Endothelial cells were pretreated with PKI (3 μ M), LY294002 (10 μ M) or PD98059 (10 μ M) for 30 min before exposure to forskolin (10 μ M). Phosphorylated ERK and Akt were determined by Western blot analyses. Endothelial cell proliferation (C), migration (D), and tube formation (E) were determined following treatment with forskolin (10 μ M) in the presence or absence of PKI (3 μ M), LY294002 (10 μ M) or PD98059 (10 μ M) as described in Fig. 1. Data shown in C to E are the mean \pm SD ($n \geq 3$). * $P < 0.05$ and ** $P < 0.01$ versus control.

results suggest that PKA, Akt, and ERK are involved in the intracellular signaling pathway of forskolin-mediated angiogenesis.

3.4. Forskolin-induced angiogenesis is also mediated by Akt-dependent eNOS/NO pathway

Since activation of Akt increases NO production by phosphorylating Ser¹¹⁷⁷ of eNOS [22], which is one of the important factors regulating angiogenesis, we examined whether forskolin increases eNOS phosphorylation and NO production. Western blot analysis revealed that forskolin increased eNOS phosphorylation in a time-dependent manner, which was effectively inhibited by co-treatment with LY294002, but not with PD98059, and was partially reduced by treatment with PKI (Fig. 4A and B). Similarly, treatment of HUVECs with forskolin elevated NO production, and this increase was markedly decreased by co-treatment with LY294002 and the NOS inhibitor NMA, but not with PD98059 (Fig. 4C and D). However, treatment with PKI partially reduced forskolin-mediated NO production. To examine a role of increased NO production in forskolin-mediated angiogenesis, HUVECs were treated with forskolin in the presence or absence of NMA, and cell migration and tube formation were measured. NMA significantly suppressed forskolin-dependent increases in migration and tube formation of HUVECs (Fig. 4E and F). We further examined the role of NO in forskolin-induced *in vivo* development of new blood vessels using an intravital microscope. Forskolin administration increased new microvessel formation in wild-type mice, and this angiogenic response was significantly lowered in eNOS-deficient mice (Fig. 4G). Co-injection with NMA significantly suppressed forskolin-induced *in vivo* angiogenesis in wild-type mice, but not in eNOS-deficient mice. These results provide strong evidence for an important role of eNOS/NO pathway in forskolin-dependent angiogenesis.

3.5. cAMP analogues mimic the angiogenic effect of forskolin

Forskolin activates guanylyl cyclase to increase the intracellular cAMP level, which appears to participate in PKA and Epac [23], resulting in the activation of various signal pathways [24,25]. We examined the effects of two membrane permeable cAMP analogues, DB-cAMP (activators of both PKA and Epac) and 8CPT-2ME-cAMP (a specific activator of Epac) on angiogenic signaling and events. Treatment with either DB-cAMP or 8CPT-2ME-cAMP promoted the phosphorylation of ERK, Akt, and eNOS (Fig. 5A). ERK phosphorylation was inhibited by PD98059, but not altered by PKI and LY924002. The phosphorylation of Akt and eNOS by these cAMP analogues was strongly inhibited by co-treatment with LY294002 alone. However, co-treatment with PKI slightly reduced DB-cAMP-induced Akt and eNOS phosphorylation, but did not alter the phosphorylation of Akt and eNOS by 8CPT-2ME-cAMP (Fig. 5A). As expected, DB-cAMP-induced HUVEC proliferation was inhibited by co-treatment with PKI and LY924002, while 8-CPT-2ME-cAMP-mediated endothelial cell proliferation was suppressed by LY924002 and PD98059, but not PKI (Fig. 5B). These results suggest that forskolin-angiogenesis requires both the PKA-dependent pathway and Epac-dependent activation of ERK and Akt/eNOS.

3.6. Forskolin up-regulates VEGF expression via PKA-dependent CREB phosphorylation

We next examined the possible involvement of VEGF, as an important angiogenic factor, in forskolin-induced angiogenesis and investigated the signal pathway for forskolin-mediated VEGF expression. Forskolin-induced HUVEC proliferation was partially suppressed by co-treatment with a VEGF-neutralizing antibody. This antibody significantly inhibited VEGF-mediated endothelial cell proliferation (Fig. 6A), indicating that

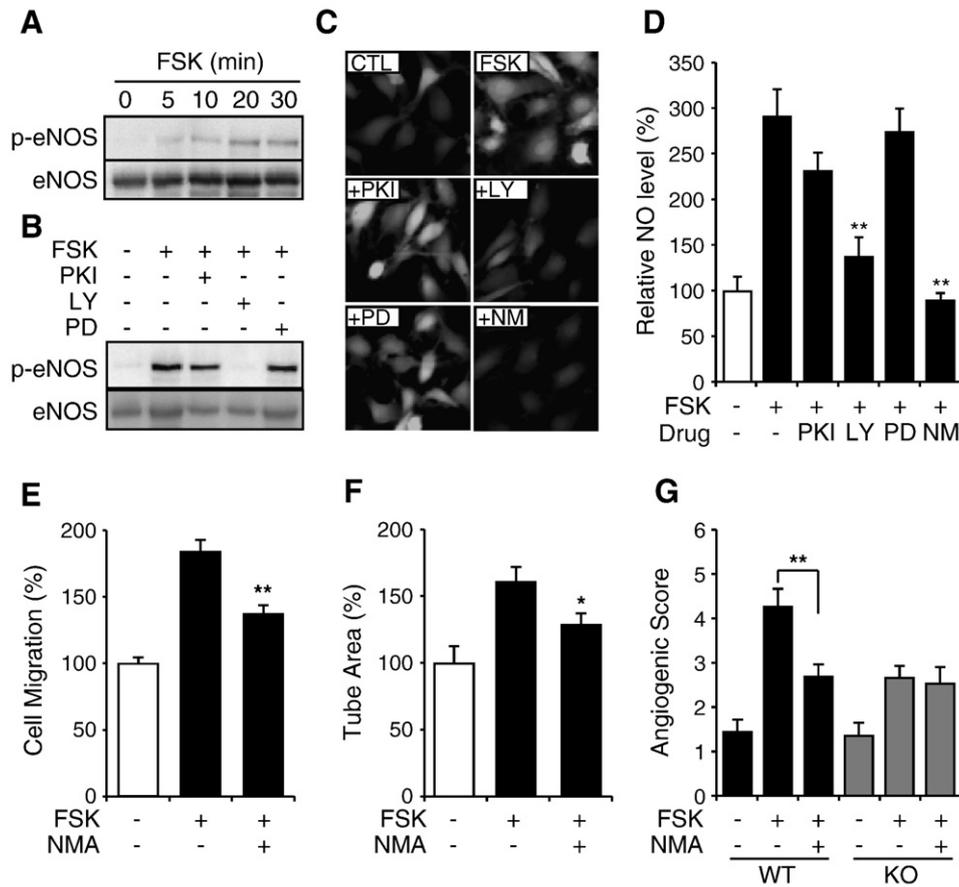


Fig. 4. The PKA-independent eNOS/NO pathway is involved in forskolin-induced angiogenesis. (A) and (B) HUVECs were incubated with forskolin (10 μ M) for the indicated times or in the presence of PKI (3 μ M), LY294002 (10 μ M) or PD98059 (10 μ M) for 30 min. eNOS phosphorylation was determined by Western blot analysis. (C) HUVECs were treated with forskolin (10 μ M) in the presence or absence of PKI (3 μ M), LY294002 (10 μ M), PD98059 (10 μ M) or NMA (1 mM) for 30 min, followed by incubation with DAF-FM/DA (5 μ M) for 30 min. Intracellular NO level was determined by a confocal microscope (\times 200), and (D) the relative level of NO was calculated from fluorescence intensities. Endothelial proliferation (E) and tube formation (F) were determined following treatment of HUVECs with forskolin (10 μ M) in the presence or absence of NMA (1 mM) for 4 h and 20–26 h, respectively, as described in the legend of Fig. 1. (G) Matrigel (100 μ l) containing forskolin (10 nmol) with or without NMA (5 mM) was put into the abdominal window of wild-type and eNOS-knockout mice. After 4 days, neovascularization was recorded after intravenous injection with FITC-labeled dextran by a Zeiss Axiovert 200 M microscope. Angiogenic activity was determined using the MetaMorph program. The relative angiogenic activity was scored from 0 (least positive) to 5 (most positive). Data shown in D to G are the mean \pm SD ($n \geq 4$). * $P < 0.05$ and ** $P < 0.01$ versus control.

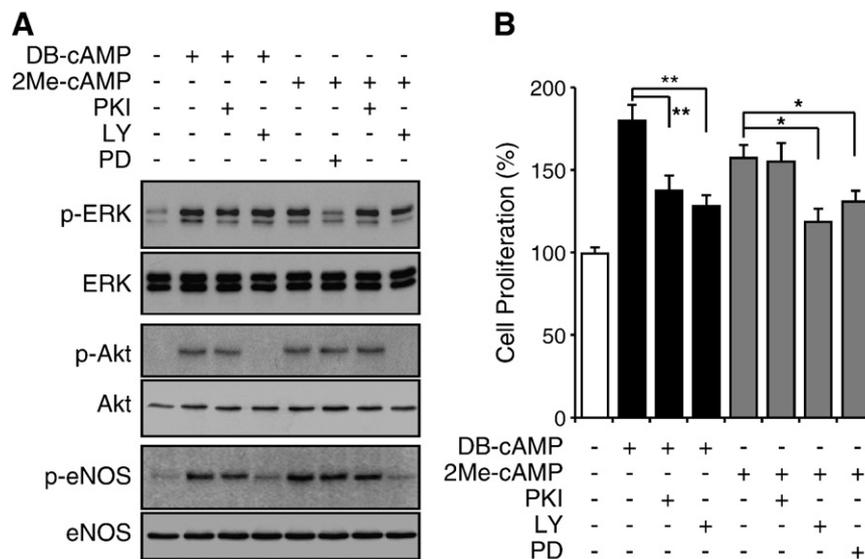


Fig. 5. 8CPT-2ME-cAMP mimics angiogenic signaling events of DB-cAMP. (A) HUVECs were incubated with 8CPT-2ME-cAMP (20 μ M) or DB-cAMP (20 μ M) in the presence or absence of PKI (3 μ M) or LY294002 (10 μ M) for 20 min. Phosphorylated ERK, Akt and eNOS were determined by Western blot analyses using specific antibodies for each protein. (B) Endothelial cell proliferation was determined following treatment with 8CPT-2ME-cAMP (20 μ M) or DB-cAMP (20 μ M) in the presence or absence of PKI (3 μ M) or LY294002 (10 μ M) by the [3 H]thymidine incorporation assay. Data shown are the mean \pm SD ($n \geq 4$). * $P < 0.05$ and ** $P < 0.01$.

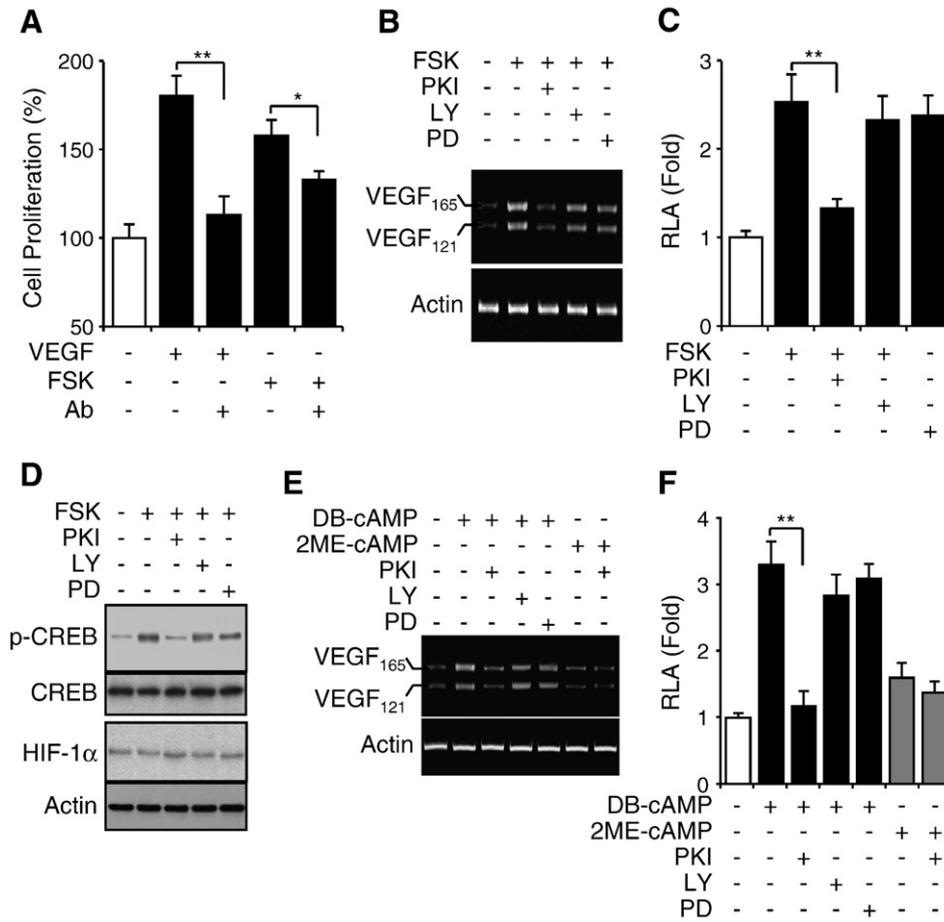


Fig. 6. Forskolin and cAMP stimulates PKA-dependent CREB phosphorylation and VEGF expression. (A) HUVECs were cultured with forskolin (10 μ M) or VEGF (10 ng/ml) following by pretreatment with a neutralizing VEGF antibody (VEGF nAb, 150 ng/ml) for 1 h. After 20 h incubation, cell proliferation was determined by the [3 H]thymidine incorporation assay. (B) HUVECs were treated with forskolin (10 μ M) in the presence or absence of PKI (3 μ M), LY294002 (10 μ M) or PD98059 (10 μ M) for 6 h. The mRNA levels of VEGF and actin were determined by RT-PCR analysis. (C) Endothelial cells were transiently transfected with the luciferase reporter plasmid containing human VEGF promoter and treated with forskolin (10 μ M) in the presence or absence of PKI (3 μ M), LY294002 (10 μ M) or PD98059 (10 μ M) for 12 h, and relative luciferase activity (RLA) in cell extracts was measured with a luminometer. (D) Cells were treated with forskolin in the presence or absence of PKI, LY294002 or PD98059 for 30 min. The levels of p-CREB and HIF-1 α were determined by Western blot analysis. (E) VEGF mRNA expression was determined following treatment of HUVECs with 8CPT-2ME-cAMP (20 μ M) or DB-cAMP (20 μ M) in the presence or absence of PKI, LY294002 or PD98059 for 6 h by RT-PCR. (F) HUVECs transfected with human VEGF promoter plasmid were treated with forskolin in the presence or absence of PKI for 12 h, and relative luciferase activity (RLA) in cell extracts was measured with a luminometer.

VEGF up-regulation is partially involved in forskolin-induced angiogenesis. To confirm the results of the VEGF-neutralizing antibody, we next examined the expression of VEGF by forskolin in HUVECs using RT-PCR analysis. Forskolin significantly increased the levels of VEGF mRNA, which was blocked by co-treatment with PKI, but not with LY294002 and PD98059 (Fig. 6B). We further examined whether forskolin regulates VEGF promoter activity using a VEGF promoter reporter plasmid containing human VEGF promoter region. Forskolin treatment resulted in a significant increase in luciferase activity (~2.6-fold) in cells transfected with the reporter plasmid compared with the untreated control, and it was significantly reduced by co-treatment with PKI, but not with LY294002 and PD98059 (Fig. 6C). This data indicates that forskolin-induced VEGF expression was associated with PKA activation, but not with Akt and ERK activation. Since the transcription factor cAMP response element binding protein (CREB), which is phosphorylated by PKA, has been implicated in mediating VEGF expression [26], we examined the effect of forskolin on CREB phosphorylation. Forskolin elevated CREB phosphorylation, was significantly inhibited by PKI, but not by LY294002 and PD98059. However, the level of HIF-1 α , which plays an important role in transcriptional expression of VEGF, was not affected in these experimental conditions (Fig. 6D). Furthermore, the level of VEGF mRNA was significantly elevated by DB-cAMP, but not by 8CPT-2ME-cAMP, and this elevation was inhibited by co-treatment with PKI, but not with

treatment of LY294002 or PD98059 (Fig. 6E). Similarly, DB-cAMP, but not 8CPT-2ME-cAMP, increased VEGF promoter activity, which was reduced by co-treatment with PKI only (Fig. 6F). These results suggest that forskolin promotes VEGF expression at the transcriptional step by activating the PKA/CREB pathway.

3.7. Specific knockdown of Epac1 reduced ERK activation and the Akt/eNOS pathway without CREB phosphorylation

Since an Epac1 inhibitor is not currently available, an RNA interference approach was used to determine the functional role of Epac in forskolin-induced signaling and angiogenesis. HUVECs have been shown to express Epac1, but not Epac2 [24]. Transfection of HUVECs with Epac1-directed siRNA resulted in the significant inhibition of Epac1 protein expression compared with cells transfected with scramble siRNA as a control (Fig. 7A). 8CPT-2ME-cAMP promoted the phosphorylation of ERK, Akt, and eNOS, but not CREB, in HUVECs transfected with control siRNA, while forskolin increased phosphorylation of all of these proteins (Fig. 7B). Forskolin elicited CREB phosphorylation, without promoting ERK, Akt, and eNOS phosphorylation, in HUVECs transfected with siRNA of Epac1, and this phosphorylation was inhibited by co-treatment with PKI. However, 8CPT-2ME-cAMP did not promote phosphorylation of all

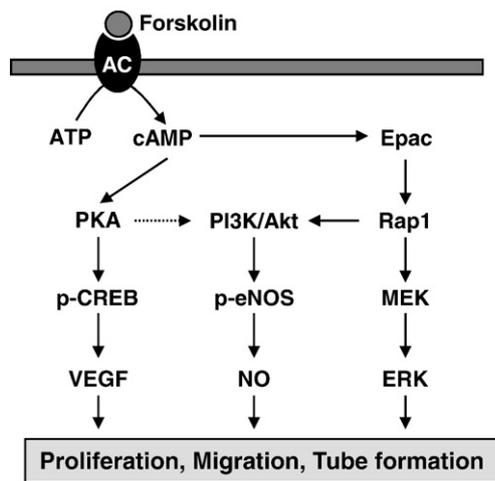


Fig. 9. The possible signal pathway of forskolin-induced angiogenesis. Activation of adenylyl cyclase by forskolin elevates the intracellular level of cAMP, which is involved in PKA-dependent CREB phosphorylation and VEGF expression as well as activation of the Epac-mediated MEK/ERK and PI3K/Akt/eNOS pathways. These signal pathways participate in proliferation, migration, and tube formation of endothelial cells. A solid arrow indicates the major activation pathway, and a dotted arrow shows the possible or minor pathway.

HUVECs by 8CPT-2ME-cAMP was suppressed to the basal level by transfection with Epac1-directed siRNA. These results suggest that Epac plays an important role in forskolin- or cAMP-induced angiogenesis by activating ERK and the Akt/eNOS pathway without regulating PKA-dependent CREB phosphorylation.

3.8. PGE₂ stimulates endothelial cell proliferation in PKA- and Epac1-dependent manners

Since PGE₂, synthesized by cyclooxygenase, has been known to be a biological activator of adenylyl cyclase and induce angiogenesis [7], we examined a role of PKA and Epac in PGE₂-induced endothelial cell proliferation. Treatment of HUVECs with PGE₂ promoted cell proliferation, and this increase was significantly, but not completely, inhibited by co-treatment with the chemical inhibitors, PKI, LY924002, PD98059, and NMA (Fig. 8A). Knockdown of Epac1 by transfection with siRNA resulted in significant suppression of PGE₂-induced HUVEC proliferation, which was further inhibited to the control level by treatment with PKI (Fig. 8B). However, PGE₂-induced proliferation in Epac1-knockdown HUVECs was not significantly altered by treatment with LY924002, PD98059, and NMA (Fig. 8B). In addition, PGE₂ increased CREB phosphorylation and VEGF mRNA expression in both control and Epac1-knockdown HUVECs, and these increases were inhibited by PKI, but not by LY924002, PD98059, and NMA (Fig. 8C and D). These results indicate that PGE₂, a biological activator of adenylyl cyclase, promoted angiogenesis via the PKA- and Epac-dependent pathways, which are identical to the signal transduction cascade of forskolin-induced angiogenesis.

4. Discussion

The present study was undertaken to elucidate the potential effect and molecular mechanism of forskolin on angiogenesis. We found that forskolin drastically increased endothelial cell proliferation, migration, and tube formation *in vitro* as well as neovascularization *in vivo*. Our data also showed that forskolin stimulated typical angiogenic signal events such as phosphorylation of ERK, Akt, and eNOS as well as elevated NO production and VEGF expression. The membrane permeable cAMP analogue DB-cAMP mimicked the angiogenic activity and intracellular events induced by forskolin. These effects were reduced by the PKA inhibitor PKI, the PI3K inhibitor LY924002, and the MEK inhibitor

PD98059. However, the angiogenic activity of the new cAMP sensor Epac-specific activator, 8CPT-2ME-cAMP, was not affected by co-treatment with PKI alone. These data indicate that forskolin and other intracellular cAMP-elevating substances increase neovascularization through PKA- and Epac-dependent signaling pathways.

Angiogenic inducers lead to an increase in endothelial cell proliferation, chemotactic motility, and tube-like structure formation by two distinct action modes such as direct activation of the angiogenic signal pathway and indirect production of angiogenic factors. The well-known angiogenic molecule VEGF is produced in a transcriptional manner by stabilization of the O₂-sensitive transcription factor HIF-1 α and phosphorylation of CREB [26,27]. We here found that forskolin and DB-cAMP, but not 8CPT-2ME-cAMP, increased CREB phosphorylation without altering HIF-1 α levels, leading to the up-regulation of VEGF expression. This event was inhibited by the inhibition of PKA activity, indicating that forskolin-mediated angiogenesis is partially elicited through the elevation of VEGF expression in a PKA/CREB-dependent manner. However, it has been also demonstrated that prostaglandin E₂, a cAMP-elevating biosubstance, induced ERK activation and endothelial cell migration, which effects were not significantly inhibited by the PKA inhibitor H89 [28]. This data suggests that ERK-dependent endothelial cell migration is mediated by the cAMP/Epac pathway, but not PKA activation. Indeed, our data showed that forskolin, DB-cAMP, and 8CPT-2ME-cAMP induced phosphorylation-dependent activation of ERK, which is closely linked to angiogenesis induced by various angiogenic factors [29,30], and this activation was inhibited by MEK activity, but not by PKA activity. These results indicate that the forskolin-induced MEK/ERK pathway is associated with Epac-dependent Rap1 activation [31]. Several lines of evidence have also showed that the PI3K/Akt pathway leads to increases in NO production via phosphorylation-dependent activation of eNOS and promotes endothelial cell survival [22]. NO production plays an important role in VEGF-induced angiogenesis including endothelial cell proliferation, migration, and tube formation [32]. Since we observed that forskolin activated the PI3K/Akt pathway in HUVECs, mainly through Epac activation (Figs. 5 and 7), the PI3K/Akt/eNOS signaling pathway may contribute to the angiogenic processes triggered by forskolin (Fig. 9). Involvement of NO in forskolin-induced angiogenesis was further confirmed using the NOS inhibitor NMA and eNOS-deficient mice (Fig. 4), indicating that forskolin stimulates angiogenesis through an increase in eNOS-mediated NO production *in vivo* and *in vitro*. These results suggest that forskolin stimulates angiogenesis through cooperative cross-talk via two distinct manners, PKA-dependent up-regulation of VEGF expression and Epac-mediated direct activation of the MEK/ERK and PI3K/Akt/eNOS pathways.

It has been well demonstrated that cAMP activates two cAMP sensor kinases, PKA and Epac [23], which may be involved in endothelial cell chemotaxis [33] and proliferation [24]. The dissociation constant (K_d) for cAMP binding to full-length Epac1 and Epac2 is 2.2–2.8 μ mol/L and ~1.2 μ mol/L, respectively [31,34,35]. However, it has been also shown that the K_d value of PKA for cAMP was 0.1–1.0 μ mol/L [34], concluding that cAMP exhibits a higher affinity for PKA than Epac. These observations suggest that PKA is preferentially activated compared with Epac in cells exposed to adenylyl cyclase-activating substances or the low dose of membrane permeable cAMP analogues including DB-cAMP and 8-Br-cAMP. However, Epac can be activated in cells where higher level of cAMP is generated. The new cAMP analogue 8CPT-2ME-cAMP exhibits a 10-fold higher affinity for Epac (K_d 2.2 μ mol/L for Epac1) than PKA (K_d 20–30 μ mol/L) [31]. The inhibition of PKA activity did not fully antagonize cAMP-mediated activation of ERK and Akt [24,25], suggesting that Epac can contribute to the ability of cAMP to promote angiogenesis. In this study, we showed that the PKA inhibitor PKI did not inhibit forskolin-mediated ERK and Akt activation and partially reduced forskolin-induced angiogenesis, indicating that both PKA and Epac play an important role in apical signaling event of forskolin- and cAMP-mediated angiogenic process. Although DB-cAMP and 8CPT-2ME-cAMP mimicked an angiogenic effect comparable to forskolin, they elicited a slightly

different signaling pathway. Indeed, we showed that DB-cAMP activated both PKA- and Epac-dependent signal pathways, and that 8CPT-3ME-cAMP exclusively activated the Epac/Rap1 pathway. Thus, both PKA and Epac participate in the angiogenic process of endothelial cells stimulated with cAMP-generating substances including forskolin.

Forskolin, a diterpene extracted from plants, exerts its biological action by binding to specific cellular receptors known as adenylyl cyclase isoforms, such as AC1 to AC8 except AC9. The enzymes responsible for the synthesis of cAMP from ATP results in the increase of cellular cAMP levels [36]. The intracellular second messenger cAMP is thought to be related to the phosphorylation and activation of multiple selective cellular substrates [37,38], including PKA and Epac [23]. Our data showed that forskolin induced the activation of Akt, eNOS, CREB, and ERK as well as increased NO production and VEGF expression, which are closely linked to angiogenesis [6,26]. Using various chemical inhibitors, we demonstrated that PKA mainly contributed to forskolin-induced VEGF expression through the phosphorylation of CREB, which is one of the important transcription factors for VEGF promoter activity. Interestingly, we also found that forskolin-induced Akt and eNOS activation and NO production were in part inhibited by treatment with PKI (Figs. 3 and 4). Similar results were reported in human aortic endothelial cells, where intracellular cAMP-elevating drugs or agents increased PKA-dependent NOS phosphorylation and NO production [39,40]. These results suggest that PKA partially contributes to the PI3K/Akt-dependent eNOS activation in HUVECs treated with forskolin (Fig. 9). However, further investigation is required to elucidate whether PKA could modulate eNOS activity through a cAMP/PKA-dependent mechanism. On the other hand, forskolin increased CREB phosphorylation and VEGF mRNA expression, but not the activation of ERK, Akt, and eNOS, in Epac1-knockdown HUVECs. Our data showed that forskolin-induced angiogenesis was inhibited by PKI treatment and specific knockdown of Epac1, indicating that the activation of the PKA- and Epac-dependent signaling cascades is involved in forskolin-induced angiogenesis. We also found that PGE₂, a biological activator of adenylyl cyclase through the ligation of EP₂ and EP₄ [7], increased endothelial cell proliferation, which was elicited by activating the PKA- and Epac-dependent pathways. These results indicate that forskolin activates the angiogenic signaling mediators, ERK, Akt, and eNOS, through an Epac-dependent manner and up-regulates VEGF expression by PKA-dependent CREB phosphorylation.

Some cellular responses, such as endothelial barrier function, endothelial cell survival, and angiogenesis, in response to cAMP analogues or cAMP-elevating substances such as forskolin and prostaglandins have been shown to be regulated exclusively by PKA or the Epac signaling pathway [6,28,39]. However, recent studies have demonstrated that both pathways are frequently interconnected in some cellular processes [41,42]. Our data showed that forskolin promoted endothelial cell proliferation, migration, and tube formation *in vitro* and neovascularization *in vivo* by elevating intracellular cAMP levels. These cellular processes were mediated by synergistic modulation between PKA-dependent VEGF expression and the Epac-dependent MEK/ERK and PI3K/Akt/eNOS pathways (Fig. 9). Therefore, our results provide comprehensive evidence of coordinated cross-talk between PKA- and Epac-mediated signal pathways in forskolin-induced angiogenesis of HUVECs.

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