The FASEB Journal express article10.1096/fj.02-1209fje. Published online August 1, 2003.

Angiogenic role of adrenomedullin through activation of Akt, mitogen-activated protein kinase, and focal adhesion kinase in endothelial cells

Won Kim,* Sang-Ok Moon,* Mi Jeong Sung,* Sung Hoon Kim,* Sik Lee,* June-No So,† and Sung Kwang Park*

*Department of Internal Medicine, Research Institute of Clinical Medicine, Chonbuk National University Medical School, Chonju, Republic of Korea; †Department of Biotechnology, Woosuk University, Chonju, Republic of Korea

Corresponding author: Sung Kwang Park, Department of Internal Medicine, Chonbuk National University Medical School, 634-18, Keum-Am dong, Chonju, 560-180, Republic of Korea, E-mail: parksk@moak.chonbuk.ac.kr

ABSTRACT

Adrenomedullin (AM) is a multifunctional peptide in human pheochromocytoma. To evaluate whether AM could be an angiogenic factor, we examined its effect on kinases and angiogenic processes. AM induced tyrosine phosphorylation of Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase1/2 (ERK1/2) by using distinct signaling pathways in human umbilical vein endothelial cells (HUVECs). AM also phosphorylated focal adhesion kinase, and phosphatidylinositol 3′-kinase inhibitor inhibited AM-induced focal adhesion kinase phosphorylation. Pretreatment with high concentrations of AM_{22-52}, a putative AM receptor antagonist, partially suppressed AM-induced phosphorylation of Akt, ERK1/2, and focal adhesion kinase. AM and vascular endothelial growth factor produced increases in DNA synthesis and migration in HUVECs. AM induced tube formation in HUVECs, and its effect was inhibited by pretreatment with phosphatidylinositol 3′-kinase inhibitor or ERK1/2 inhibitor. AM induced sprouting in porcine pulmonary arterial endothelial cells and promoted neovessel formation in a mouse Matrigel plug assay. Inhibitors of phosphatidylinositol 3′-kinase and ERK1/2 inhibited AM-induced endothelial sprouting in vitro and angiogenesis in vivo. AM exerts angiogenic activity through activation of Akt, MAPK, and focal adhesion kinase in endothelial cells.

Key words: angiogenesis • ERK

Human adrenomedullin (AM) is a 52-amino acid peptide that was discovered in pheochromocytoma cells (1). Although AM was discovered in malignant tissue arising from adrenal medulla, AM mRNA has been shown to be highly expressed in normal tissues such as cardiac atrium and ventricle, aorta, kidney, lung, and uterus as well as in adrenal gland (2, 3). AM protein is also highly expressed in adrenal gland, atrium, and lung (2). However, the concentration of immunoreactive AM in aorta and ventricle was <5% of that in adrenal gland, even though a high level of AM mRNA was found in these circulatory system tissues (2). This fact suggested that AM synthesized in these tissues may be rapidly and

constitutively secreted into the blood or function as an autocrine or paracrine regulator (4). Indeed, it has been demonstrated that vascular endothelial cells and vascular smooth muscle cells actively synthesize and secrete AM (5, 6). In addition, the functional AM receptor, composed of calcitonin receptor-like receptor (CRLR) and the receptor activity-modifying protein (RAMP) 2 or RAMP 3 is found in vascular endothelial cells (7, 8). Thus, AM is actively synthesized and secreted from vascular endothelial and smooth muscle cells and has an autocrine and paracrine role in the vascular system.

It is well established that AM has multiple functions. Intravenous injection of AM causes a potent and long-lasting hypotensive effect in anesthetized rats in vivo (1). AM also decreases vascular endothelial cell apoptosis and acts as an antiproliferative factor for vascular smooth muscle cells (9, 10). AM inhibites smooth muscle cell migration (11). Thus, AM had functions as a regulator in cell differentiation, survival, and growth in the vascular system (12).

AM is also expressed in the female reproductive tract, especially in the ovarian granulosa cells, uterine epithelial cells, stromal cells, endometrial endothelial cells, uterine smooth muscle cells, and placenta cells (13). The AM level in uterus was shown to change in accordance with reproductive cycles (14). The AM mRNA levels increase in the corpus luteum during the mid-luteal phase and are maintained during early pregnancy (15). AM also promotes growth of endometrial endothelial cells (16). Because angiogenesis is actively associated with follicular development, corpus luteum formation, and uterine endometrial proliferation, these findings suggest that the high expression level of AM in uterus and placenta is related to angiogenesis and vasculogenesis in the reproductive tract. Recently, it was reported that AM was induced by tamoxifen and had an angiogenic function in normal uterine endometrium (17).

In tumor cells, inflammation and hypoxia increase AM expression (18). The elevated expression of AM is associated with tumor neovascularization in xenografted endometrial tumors and renal cell carcinoma (19, 20). AM is also a tumor cell survival factor underlying human carcinogenesis (18). Thus, AM may have a significant role in tumor angiogenesis.

The role of AM in vascular development was demonstrated by an AM gene knockout mouse (21). In knockout mice, AM is indispensable for vascular morphogenesis during embryonic development (22). Targeted null mutation of the AM gene is lethal in utero, with extreme hydrops fetalis and cardiovascular abnormalities (21, 22). Although knockouts of the gene encoding AM result in embryonic lethality in mice due to an absence of blood vessel formation, the biological activity and the signal pathway of AM have not yet been described.

Thus, it is known that AM has important roles in tumor angiogenesis, vascular development, and angiogenesis of the female reproductive system. However, the signaling pathway used by AM in vascular angiogenesis has not been reported. In this report, we investigated the angiogenic effect of AM on endothelial cells and the possible mechanisms of AM-induced angiogenesis in vascular endothelial cells. We found that AM-induced angiogenesis in an in vivo experiment and that it induces sprouting through endothelial cell proliferation, migration, and tube formation in cultured endothelial cells. AM-induced angiogenesis occurred through phosphatidylinositol 3′-kinase (PI 3′-kinase)/Akt, extracellular signal-regulated kinase (ERK), and tyrosine phosphorylation of focal adhesion kinase ($p125^{FAK}$). Therefore, we propose that AM induces angiogenesis in endothelial cells through the activation of Akt, ERK, and $p125^{FAK}$.

MATERIALS AND METHODS

Materials and cell culture

Fetal bovine serum (FBS) was purchased from Life Technologies (Gaithersburg, MD). Collagenase type II and elastase were purchased from Worthington Biomedical (Freehold, NJ). AM, AM_{22-52}, gelatin, antibiotics, antimycotics, monoclonal mouse anti-human factor VIII-related antigen, M199, wortmannin, Matrigel, microcarrier beads, and trypsin-EDTA were from Sigma-Aldrich (St. Louis, MO). Recombinant human vascular endothelial growth $factor_{165}$ (VEGF) was purchased from R&D Systems (Minneapolis, MN). Wortmannin and PD98059 were obtained from Calbiochem (San Diego, CA). Antibodies for phospho-specific ERK1/2 (Thr202/Tyr204), Akt, and phospho-specific Akt (Ser473) were purchased from New England Biolabs (Beverly, MA). Antibodies for $p125^{FAK}$ and phospho-specific $p125^{FAK}$ (Tyr397) were received from Upstate Biotechnology (Lake Placid, NY). Boyden chambers were purchased from Neuroprobe (Cabin John, MD). Cell culture products and most other biochemical reagents were purchased from Sigma-Aldrich, unless otherwise specified. The primary cultured cells used in this study were between passage 2 and 3. The porcine pulmonary artery endothelial cells (PPAECs) and human umbilical vein endothelial cells (HUVECs) were isolated using methods described previously (23, 24).

Biochemical analyses

HUVECs were incubated in M199 medium with 1% FBS for 16 h before addition of AM. To measure the activity of mitogen-activated protein kinase (MAPK), and the phosphorylation of Akt and $p125^{FAK}$, HUVECs were treated with AM for the indicated times and doses. The treated HUVECs were washed two times with phosphate-buffered saline (PBS), dissolved in sample buffer (50 mM Tris-HCl, [pH 7.5], 100 mM NaCl, 0.1% SDS, 1% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin), boiled, separated by SDS-PAGE, and transferred to nitrocellulose membrane. The membranes were treated, and the phosphorylation levels were analyzed according to the manufacturer's protocols. All signals were visualized and analyzed by densitometric scanning (Image Analyzer, Fullerton, CA).

Assays of DNA synthesis and migration

The DNA synthetic activity of HUVECs was measured by a [^{3}H] thymidine incorporation assay described previously (25). In brief, HUVECs were plated onto gelatinized 24-well plates at a density of 2×10^4 cells/well in M199 medium supplemented with 20% (vol/vol) FBS. After 18 h in culture medium with 1% FBS, the medium was changed and growth factors or control buffer were added in M199 medium supplemented with 2% FBS. After 24 h, fresh medium and growth factors were added and the cells were incubated for an additional 24 h. Cells were labeled with [^{3}H] thymidine (25 mCi/mmol; Amersham, q2lesbury, UK) for 48 h before the assay. After labeling, unincorporated [^{3}H] methylthymidine was removed by washing with 10% trichloroacetic acid. Incorporated [^{3}H] methylthymidine was extracted in 0.2 M NaOH and 0.1% SDS at 37°C for 30 h. The values samples were counted with a liquid scintillation counter (Beckman Instruments, Fullerton, CA).

The migration assay with HUVECs was performed using a modified Boyden chamber as described previously (26). Growth factors in M199 containing 1% bovine serum albumin (BSA) were placed in the bottom wells of the chamber. Polycarbonate filters with 8-μm pores (Poretics, Livermore, CA) were coated with 0.2% gelatin and placed between the test substances and the upper chambers. Cells were trypsinized, washed twice in M199, and resuspended in M199 containing 1% BSA. We placed 2×10^5 cells into each well in the upper chamber and then incubated them for 12 h at 37°C in a humidified chamber with 5% CO_2. After incubation, the nonmigrated cells were removed from the upper side of the filters with a cotton ball. The filters were fixed with methanol, mounted onto microscope slides, and stained with Diff-Quik solution. The migrated cells were counted at 100× magnification, using a microscope. VEGF (10 ng/ml) was used as a positive control.

Sprouting assays

The sprouting assay with PPAECs was performed as described previously (24). In brief, PPAECs were grown to confluence on a culture dish. The placement of microcarrier beads (175 μm diameter) onto a confluent monolayer of PPAECs for 2–3 days produces beads covered by a confluent monolayer of cells. Beads were placed in a 6 mg/ml fibrinogen gel containing 2.0% heat-inactivated FBS. Fibrin gels were incubated in Dulbecco's modified Eagle's medium (DMEM) with a daily addition of control buffer, AM (10^{-7} mol/l), VEGF (10 ng/ml), or AM plus wortmannin (30 nmol/l) and PD98059 (2 μmol/l). After 2 days, two independent, blinded investigators counted the number of endothelial sprouts. To quantify the degree of endothelial sprouting, two methods were used. Using an inverted microscope (Zeiss, Gottingen, Germany), we counted the number of endothelial sprouts with length exceeding the diameter of the microcarrier bead (long endothelial sprouts). We also assessed the total number of endothelial sprouts visible (total endothelial sprouts).

In vitro and in vivo angiogenesis assays

In vitro tube formation was performed in three-dimensional cultures of HUVECs on gel matrices with rat tail collagen (type I; Roche Molecular Biochemicals, Indianapolis, IN) at a final concentration of 1.75 mg/ml. The collagen solution was prepared in M199 culture medium with fibronectin (90 mg/ml), 150 mM HEPES, and sodium bicarbonate, neutralizing the pH by the addition of 1 M NaOH. HUVECs were added immediately to a final concentration of 2×10^6 cells/ml. The cell-collagen mixture (0.5 ml each) was added to 24-well dishes and placed in a humidified CO_2 incubator at 37°C for 30 min, allowing the gel to solidify. The medium without FBS was supplemented with or without AM and the indicated kinase inhibitors. AM and kinase inhibitor were replenished every 24 h. Tube formation in each well was monitored, and photos were taken at 48 h using an inverted microscope (Zeiss).

A Matrigel plug assay was performed as described previously (27). In brief, C57/BL6 mice were injected s.c. with 0.5 ml of Matrigel with heparin (50 U/ml) that contained control buffer, AM, or AM plus wortmannin (30 nmol/l) and PD98059 (2 μmol/l). All three gels were injected into the same animal at different locations on the flanks in order to avoid individual variation. Each portion of injected Matrigel rapidly formed a single solid gel plug. After 2 wk, mice were killed, and the Matrigel plugs were recovered, fixed with 4% paraformaldehyde in PBS, embedded in paraffin, sectioned, and stained with Mason-Trichrome solution for microscopic observation. To

quantify the formation of functional neovessels in Matrigel, the amount of hemoglobin in each plug was assayed according to the manufacturer's protocol (Drabkin reagent kit 525, Sigma-Aldrich).

Data analyses

Data are expressed as mean ±SD. Statistical significance was tested using one-way ANOVA followed by the Student-Newman-Keuls test. Statistical significance was set at $P<0.05$.

RESULTS

AM induces Akt, ERK1/2, and p125^{FAK} phosphorylation

Activation of Akt, ERK, and p125^{FAK} in endothelial cells plays an important role in angiogenesis (24, 28, 29). To determine Akt activity, we measured the phosphorylation of Akt. AM (10^{-7} mol/l) increased Akt phosphorylation as early as 10 min and produced a maximal effect at 30 min (Fig. 1A). The maximum mean increase in Akt phosphorylation was 6.2-fold. AM (10^{-11}–10^{-7} mol/l) increased Akt phosphorylation in a dose-dependent manner (Fig. 1D). To determine MAPK activity, we measured the phosphorylation of two MAPKs, ERK1 (p44^{MAPK}) and ERK2 (p42^{MAPK}). AM (10^{-7} mol/l) increased ERK1/2 phosphorylation as early as 5 min and produced a maximal effect at 10 min (Fig. 1B). These effects declined and returned to control levels at 30 min. The maximum mean increase in ERK2 phosphorylation was 7.1-fold. AM (10^{-11}–10^{-7} mol/l) also increased ERK1/2 phosphorylation in a dose-dependent manner (Fig. 1E). To examine p125^{FAK} activity, we measured the tyrosine phosphorylation of p125^{FAK}. AM (10^{-7} mol/l) induced p125^{FAK} phosphorylation as early as 5 min and produced a maximal effect at 10 min (Fig. 1C). These effects declined but continued to be higher than control levels at up to 15 min. The maximum mean increase in p125^{FAK} was 3.7-fold. Thus, the AM-induced phosphorylation of p125^{FAK} and ERK1/2 peaked at the same time. AM (10^{-11}–10^{-7} mol/l) also induced p125^{FAK} phosphorylation in a dose-dependent manner (Fig. 1F).

AM mediates phosphorylation of Akt and ERK, using distinct signaling pathways, and the phosphorylation effect of AM is partially inhibited by AM$_{22-52}$

The Western blot analysis in Figure 1 demonstrated AM-dependent activation of Akt and ERK1/2 in HUVECs. To rule out any crosstalk between the two pathways activated by AM (10^{-7} mol/l), we investigated the effect of the PI3′-kinase inhibitor, wortmannin on ERK1 and ERK2 phosphorylation and MEK1/2 inhibitor, PD98059 on Akt phosphorylation. Although 30 nmol/l of wortmannin was sufficient to inhibit AM-induced phosphorylation of Akt (Fig. 2A), this dosage had no effect on ERK1 and ERK2 phosphorylation (Fig. 2B). On the other hand, the MEK1/2 inhibitor, PD98059 significantly inhibited AM-induced phosphorylation of ERK1 and ERK2 (Fig. 2B) but had no effect on the phosphorylation of Akt (Fig. 2A). We conclude, therefore, that AM mediates phosphorylation of Akt and MAPK, using distinct signaling pathways. In addition, we tested the effect of these inhibitors on AM-induced p125^{FAK} phosphorylation. Pretreatment of PI 3′-kinase-specific inhibitor, wortmannin (30 nmol/l), almost totally abolished the AM-induced p125^{FAK} phosphorylation (Fig. 2C). However, pretreatment of PD98059 (50 nmol/l) only partially reduced p125^{FAK} phosphorylation (Fig. 2C). This finding

suggests that AM-induced $p125^{FAK}$ phosphorylation is mainly regulated by a PI 3′-kinase dependent pathway.

AM_{22-52} is a putative inhibitor of the AM receptor. We tested the effect of AM_{22-52} (10^{-10}, 10^{-8}, and 10^{-6} mol/l) in HUVECs. Pretreatment with AM_{22-52} at 10^{-10} mol/l slightly decreased AM-induced phosphorylation of ERK1/2, Akt, or $p125^{FAK}$, and pretreatment of AM_{22-52} at higher concentrations (10^{-6} mol/l) partially suppressed AM-induced phosphorylation of the three kinases (Fig. 2D, 2E, and 2F). These results support the idea that phosphorylation of Akt, ERK1/2, and $p125^{FAK}$ by AM is partially mediated through AM_{22-52} -sensitive receptors.

AM increases DNA synthesis and migration in endothelial cells

MAPK and Akt participate in protein kinase cascades that play critical roles in the regulation of cell growth and migration (28–31). Because ERK1/2 and Akt activation might be involved in the proliferation and migration in endothelial cells, we examined the effect of AM on endothelial cell DNA synthesis and migration. The degree of endothelial cell DNA synthesis was estimated by a [^{3}H] thymidine incorporation assay. After a 48-h treatment, AM (10^{-7}mol/l) and VEGF (10 ng/ml) increased [^{3}H] thymidine incorporation ~1.4-fold and 1.7-fold, respectively (Fig. 3A). These data demonstrated that AM increased DNA synthesis in a dose-dependent manner.

To examine whether AM affects endothelial cell migration, we pretreated HUVECs with these compounds and placed them into a Boyden chamber in the absence or presence of AM (10^{-11}–10^{-7} mol/l). Aftr a 12-h treatment, AM (10^{-7} mol/l) increased the migration of HUVECs approximately fourfold, while VEGF (10 ng/ml) increased migration approximately sixfold (Fig. 3B). These results indicated that AM stimulated endothelial migration in a dose-dependent manner.

AM induces tube formation by endothelial cells in collagen gel

Endothelial cells form as a precursor to blood vessel formation (31). Well-known endothelial growth factors, including VEGF and angiopoietin-1, increase tube formation of endothelial cells through enhanced migration (24, 32, 33). Because our data indicated that AM is a relatively strong enhancer of migration, we examined the effect of AM on tube formation of HUVECs in type I collagen gel. After a 48-h treatment, AM (10^{-7} mol/l) and VEGF (10 ng/ml) increased tube formation ~3.1-fold and 4.0-fold, respectively (Fig. 4A, 4B). MEK1/2 inhibitor, PD98059, and PI 3′-kinase inhibitor, wortmannin, significantly suppressed AM-induced tube formation (Fig. 4A, 4B). The inhibitors alone mildly suppressed basal tube formation at 48 h, but not at a statistically significant level (Fig. 4B). These data suggest that AM-induced tube formation in endothelial cells is mediated through PI 3′-kinase and the MEK1/2 pathway.

AM induces sprouting in PPAECs

Sprouting, an essential step in initial phase of angiogenesis, requires cell migration, cell proliferation, and tube formation (31, 34). Because AM induced cell migration, cell proliferation, and tube formation, we evaluated the effect of AM on sprouting in PPAECs. We used two assays in PPAECs to quantify the degree of sprouting caused by AM. After 48 h, AM (10^{-7} mol/l) increased the total number of total endothelial spouts and long endothelial sprouts ~2.9-fold and

2.5-fold, respectively (Fig. 5A, 5B). As a positive control, VEGF (10 ng/ml) increased the total number of endothelial spouts and long endothelial sprouts ~4.0-fold and 3.5-fold, respectively (Fig. 5A, 5B). Thus, the relative potency of VEGF in endothelial sprouting formation was higher than that produced by AM. MEK1/2 inhibitor, PD98059, or PI 3′-kinase inhibitor, wortmannin, partially suppressed AM-induced sprouting (Fig. 5A, 5B). The inhibitors alone mildly suppressed basal sprouting at 48 h, but not at a statistically significant level (data not shown). The relative inhibitory potency, judged by the total number of endothelial sprouts, was higher for wortmannin than for PD98059 (Fig. 5B). However, the relative inhibitory potency evaluated by the number of long endothelial sprouts was similar for the two inhibitors (Fig. 5B). The combination of PD98059 and wortmannin completely inhibited AM-induced endothelial sprouting formation in PPAECs. Although AM-induced sprout formation is mediated through PI 3′-kinase and the MEK1/2 pathway, the major signaling is through a PI 3′-kinase-dependent pathway.

AM promotes angiogenesis in vivo

To determine whether AM is capable of promoting angiogenesis in vivo, we performed an established in vivo angiogenesis assay, the mouse Matrigel plug assay. Histochemical staining indicated that Matrigel containing AM (2×10^{-7} mol/l) produced more neovessels within gels than Matrigel containing control buffer (Fig. 6A). Matrigel containing AM (2×10^{-7} mol/l) had more hemoglobin content than Matrigel containing control buffer (Fig. 6B). Addition of PD98059 plus wortmannin suppressed these AM-induced angiogenic activities (Fig. 6A, 6B). When quantified by measurement of hemoglobin in the Matrigels, the angiogenic activity of AM was comparable to that of 20 ng/ml of VEGF (data not shown). Thus, AM has an angiogenic activity in vivo through activation of ERK and Akt.

DISCUSSION

In this report, we demonstrate that AM induces angiogenesis through intracellular Akt, MAPK, and $p125^{FAK}$ activation in endothelial cells. Accordingly, AM promoted endothelial DNA synthesis, migration, and tube formation, which are the essential steps for angiogenesis. Indeed, AM promoted sprouting in vitro and neovessel formation in vivo in gel plugs. Thus, we demonstrate here the signaling pathways of AM in angiogenesis (Fig. 1, 2).

Activation of MAPK and Akt in endothelial cells is a crucial intracellular signaling step for angiogenesis (29, 30). Growth factors such as VEGF or angiopoietin-1 bind to their receptor tyrosine kinases in endothelial cells and induce angiogenesis through activation of these kinases (24, 28–30). Our Western blot analyses indicated that AM induced phosphorylation of Akt and ERK in HUVECs, and AM fragment, $AM_{22\text{-}52}$ partially suppressed AM-induced phosphorylation of Akt and ERK. Therefore, AM-induced activation of Akt and ERK could occur, at least in part, through an $AM_{22\text{-}52}$-sensitive receptor. Our Western blot data also suggest that there is no crosstalk between AM-induced Akt and ERK pathways. Thus, AM mediates phosphorylation of Akt and ERK, using distinct signaling pathways. Using thin-layer chromatography, we also found that AM (10^{-8} mol/l) caused maximal activation of PI 3′-kinase activity within 3–5 min in HUVECs (data not shown). Thus, AM-induced Atk activation can be mediated through activation of PI 3′-kinase.

A functional AM receptor consists of two proteins, CRLR and the RAMPs, that couple the receptor to the cellular signal transduction pathway. Because CRLR is a G protein-coupled receptor, we wanted to know whether the Gαi-protein is involved in the PI 3′-kinase pathway. HUVECs were pretreated with a Gαi/o protein inhibitor, pertussis toxin (200, 400, and 800 ng/ml), or control buffer for 2 h. Then the cells were incubated in medium containing 1% serum and AM (10^{-7} mol/l) for 3 min. The PI 3′-kinase assay was performed. Pertussis toxin reduced AM-induced phosphorylation of PI 3′-kinase activity (AM alone, 5.7±1.4; AM + 200 ng/ml pertussis toxin, 3.5±0.7; AM + 400 ng/ml pertussis toxin, 1.9±0.5; AM + 800 ng/ml pertussis toxin, 1.6±0.5; control, 1.0±0.0). These results suggested that AM-induced phosphorylation of PI 3′-kinase occurs through a G protein-coupled receptor, probably CRLR.

In the initial phase of angiogenesis and neovascularization, the sprouting of endothelial cells is an essential step (31). This process requires cell migration, cell proliferation, and tube formation (31). Our results showed that AM induced proliferation, migration, and tube formation in endothelial cells in vitro (Fig. 4, 5). We also confirmed that AM induced sprouting from endothelial cells. Furthermore, we demonstrated that AM-induced sprout formation was mediated through PI 3′-kinase and MEK1/2 pathway.

A member of the nonreceptor protein tyrosine kinases, $p125^{FAK}$, has been implicated in the regulation of the actin cytoskeleton during migration (35). $p125^{FAK}$ is activated by tyrosine phosphorylation, which is induced by growth factors (24, 36, 37). Because AM has a migratory effect in endothelial cells, we examined whether AM could stimulate $p125^{FAK}$. In this study, we found that AM induces tyrosine phosphorylation of $p125^{FAK}$ in a time- and concentration-dependent manner in endothelial cells (Fig. 1). Next, we found that the PI 3′-kinase inhibitors completely inhibit AM-stimulated tyrosine phosphorylation of $p125^{FAK}$ in HUVECs. These results suggest that PI 3′-kinase is part of the signal transduction pathway inducing the tyrosine phosphorylation of $p125^{FAK}$. Because $p125^{FAK}$ has been implicated in migration, all of these results suggest that the migratory effect of AM in endothelial cells may be mediated through AM-induced tyrosine-phosphorylated $p125^{FAK}$ by a PI 3′-kinase-dependent pathway.

It is well known that endothelial cell survival is an essential prerequisite for DNA synthesis and migration. Previously, Kato et al. (9) reported that AM is an endothelial cell survival factor. We also demonstrated that the antiapoptotic effect of AM is associated with Akt pathway activation (23). Because AM is a survival factor for endothelial cells, AM could have an angiogenic role in vascular endothelial cells. To produce sprouting in response to AM stimulation in an in vitro fibrin gel, endothelial cells must release proteinases to degrade extracellular matrix for their migration (38, 39). One family of such enzymes is the matrix metalloproteinases (MMPs) (40). We found that AM stimulated the hydrolytic activities of plasmin, MMP-2, and MMP-9 in HUVECs measured by gelatin zymography, and furthermore that these induced activities were inhibited by PI 3′-kinase inhibitors (data not shown). Thus, these two findings give additional evidence that AM is an angiogenic factor in vitro.

We wondered whether the effect of AM might rely on an indirect effect through some other angiogenic factor. Thus, we examined the changes in VEGF-A and angiopoietin-2 mRNAs after treatment with AM (10^{-7} mol/l). An RNase protection assay in HUVECs showed that AM did not significantly change the mRNA expression of VEGF-A or angiopoietin-2 for 0, 2, 4, and 6 h

(data not shown). Thus, these data suggested that AM did not change VEGF-A and angiopoietin-2.

We confirmed the angiogenic activity of AM by performing an in vivo mouse Matrigel plug assay. AM promoted neovessel formation in the Matrigel plug, and we noted that these newly synthesized vessels participated actively in the circulation of blood cells. Moreover, our pharmacological inhibition study indicated that the activation of MAPK and Akt by AM is crucial in endothelial tube formation and neovessel formation in the Matrigel plug. These results suggest that the activities and pathways of AM found in our in vitro experiments are likely to be valid in vivo as well. Taken together, these data show that the AM-induced angiogenic process may be accomplished by and mainly mediated through intracellular PI 3′-kinase/Akt -$p125^{FAK}$ and Ras/Raf/MEK/ERK1/2 activation (Fig. 7).

In tumor cells, AM may have a significant role in angiogenesis. Because angiogenesis is an essential process in tumor-host interactions for tumor growth, maintenance, and metastasis, finding ways to regulate the action of AM may provide a new avenue for finding anticancer therapeutics.

Until recently, fibroblast growth factor, platelet-derived growth factor, VEGF, and angiopoietin-1 were known to have profound angiogenic effects in endothelial cells. In this study, we demonstrated that AM is a new angiogenic factor and its signaling is mediated through activation of Akt, MAPK, and focal adhesion kinase in endothelial cells. These results suggest that AM is a candidate for therapeutic angiogenesis or vasculogenesis in ischemic disease. Further studies are needed to assess the pathogenic role of AM in vascular diseases, therapeutic endothelial survival, and the counteractive or additive effect of AM with other angiogenic factors.

ACKNOWLEDGMENTS

We thank Ms. Jennifer Macke for assistance in editing the text. This work was supported by a grant from the basic research program of Korea Science and Engineering Foundation (R05-2003-000-11393-0).

REFERENCES

1. Kitamura, K., Kangawa, K., Kawamoto, M., Ichiki, Y., Nakamura, S., Matsuo, H., and Eto, T. (1993) Adrenomedullin: a novel hypotensive peptide isolated from human pheochromocytoma. *Biochem. Biophys. Res. Commun.* **192,** 553–560

2. Kitamura, K., Kangawa, K., and Eto, T. (2002) Adrenomedullin and PAMP: discovery, structures, and cardiovascular functions. *Microsc. Res. Tech.* **57,** 3–13

3. Sakata, J., Shimokubo, T., Kitamura, K., Nishizono, M., Iehiki, Y., Kangawa, K., Matsuo, H., and Eto, T. (1994) Distribution and characterization of immunoreactive rat adrenomedullin in tissue and plasma. *FEBS Lett.* **352,** 105–108

4. Bean, A. J., Zhang, X., and Hokfelt, T. (1994) Peptide secretion: what do we know? *FASEB J.* **8,** 630–638

5. Sugo, S., Minamino, N., Kangawa, K., Miyamoto, K., Kitamura, K., Sakata, J., Eto, T., and Matsuo, H. (1994) Endothelial cells actively synthesize and secrete adrenomedullin. *Biochem. Biophys. Res. Commun.* **201,** 1160–1166

6. Sugo, S., Minamino, N., Shoji, H., Kangawa, K., Kitamura, K., Eto, T., and Matsuo, H. (1994) Production and secretion of adrenomedullin from vascular smooth muscle cells: augmented production by tumor necrosis factor-alpha. *Biochem. Biophys. Res. Commun.* **203,** 719–726

7. McLatchie, L. M., Fraser, N. J., Main, M. J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M. G., and Foord, S. M. (1998) RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature* **393,** 333–339

8. Kamitani, S., Asakawa, M., Shimekake, Y., Kuwasako, K., Nakahara, K., and Sakata, T. (1999) The RAMP2/CRLR complex is a functional adrenomedullin receptor in human endothelial and vascular smooth muscle cells. *FEBS Lett.* **448,** 111–114

9. Kato, H., Shichiri, M., Marumo, F., and Hirata, Y. (1997) Adrenomedullin as an autocrine/paracrine apoptosis survival factor for rat endothelial cells. *Endocrinology* **138,** 2615–2620

10. Kano, H., Kohno, M., Yasunari, K., Yokokawa, K., Horio, T., Ikeda, M., Minami, M., Hanehira, T., Takeda, T., and Yoshikawa, J. (1996) Adrenomedullin as a novel antiproliferative factor of vascular smooth muscle cells. *J. Hypertens.* **14,** 209–213

11. Horio, T., Kohno, M., Kano, H., Ikeda, M., Yasunari, K., Yokokawa, K., Minami, M., and Takeda, T. (1995) Adrenomedullin as a novel antimigration factor of vascular smooth muscle cells. *Circ. Res.* **77,** 660–664

12. Hinson, J. P., Kapas, S., and Smith, D. M. (2000) Adrenomedullin, a multifunctional regulatory peptide. *Endocr. Rev.* **21,** 138–167

13. Minamino, N., Kikumoto, K., and Isumi, Y. (2002) Regulation of adrenomedullin expression and release. *Microsc. Res. Tech.* **57,** 28–39

14. Cameron, V. A., Autelitano, D. J., Evans, J. J., Ellmers, L. J., Espiner, E. A., Nicholls, M. G., and Richards, A. M. (2002) Adrenomedullin expression in rat uterus is correlated with plasma estradiol. *Am. J. Physiol. Endocrinol. Metab.* **282,** E139–E146

15. Abe, K., Minegishi, T., Ibuki, Y., Kojima, M., and Kangawa, K. (2000) Expression of adrenomedullin in the human corpus luteum. *Fertil. Steril.* **74,** 141–145

16. Nikitenko, L. L., MacKenzie, I. Z., Rees, M. C., and Bicknell, R. (2000) Adrenomedullin is an autocrine regulator of endothelial growth in human endometrium. *Mol. Hum. Reprod.* **6,** 811–819

17. Zhao, Y., Hague, S., Manek, S., Zhang, L., Bicknell, R., and Rees, M. C. (1998) PCR display identifies tamoxifen induction of the novel angiogenic factor adrenomedullin by a non estrogenic mechanism in the human endometrium. *Oncogene* **16,** 409–415

18. Cuttitta, F., Pio, R., Garayoa, M., Zudaire, E., Julian, M., Elsasser, T. H., Montuenga, L. M., and Martinez, A. (2002) Adrenomedullin functions as an important tumor survival factor in human carcinogenesis. *Microsc. Res. Tech.* **57,** 110–119

19. Oehler, M. K., Hague, S., Rees, M. C., and Bicknell, R. (2002) Adrenomedullin promotes formation of xenografted endometrial tumors by stimulation of autocrine growth and angiogenesis. *Oncogene* **21,** 2815–2821

20. Fujita, Y., Mimata, H., Nasu, N., Nomura, T., Nomura, Y., and Nakagawa, M. (2002) Involvement of adrenomedullin induced by hypoxia in angiogenesis in human renal cell carcinoma. *Int. J. Urol.* **9,** 285–295

21. Caron, K. M., and Smithies, O. (2001) Extreme hydrops fetalis and cardiovascular abnormalities in mice lacking a functional adrenomedullin gene. *Proc. Natl. Acad. Sci. USA* **98,** 615–619

22. Hay, D. L., and Smith, D. M. (2001) Knockouts and transgenics confirm the importance of adrenomedullin in the vasculature. *Trends Pharmacol. Sci.* **22,** 57–59

23. Kim, W., Moon, S. O., Sung, M. J., Kim, S. H., Lee, S., Kim, H. J., Koh, G. Y., and Park, S. K. (2002) Protective Effect of adrenomedullin in mannitol-induced apoptosis. *Apoptosis* **7,** 527–536

24. Kim, I., Kim, H. G., Moon, S. O., Chae, S. W., So, J. N., Koh, K. N., Ahn, B. C., and Koh, G. Y. (2000) Angiopoietin-1 induces endothelial cell sprouting through the activation of focal adhesion kinase and plasmin secretion. *Circ. Res.* **86,** 952–959

25. Lee, M. S., Moon, E. J., Lee, S. W., Kim, M. S., Kim, K. W., and Kim, Y. J. (2001) Angiogenic activity of pyruvic acid in in vivo and in vitro angiogenesis models. *Cancer Res.* **61,** 3290–3293

26. Kim, H. S., Shin, H. S., Kwak, H. J., Cho, C. H., Lee, C. O., and Koh, G. Y. (2003) Betacellulin induces angiogenesis through activation of mitogen-activated protein kinase and phosphatidylinositol 3′-kinase in endothelial cell. *FASEB J.* **17,** 318–320

27. Kim, Y. M., Kim, Y. M., Lee, Y. M., Kim, H. S., Kim, J. D., Choi, Y., Kim, K. W., Lee, S. Y., and Kwon, Y. G. (2002) TNF-related activation-induced cytokine (TRANCE) induces angiogenesis through the activation of Src and phospholipase C (PLC) in human endothelial cells. *J. Biol. Chem.* **277,** 6799–6805

28. Takahashi, T., Ueno, H., and Shibuya, M. (1999) VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. *Oncogene* **18,** 2221–2230

29. Wu, L. W., Mayo, L. D., Dunbar, J. D., Kessler, K. M., Baerwald, M. R., Jaffe, E. A., Wang, D., Warren, R. S., and Donner, D. B. (2000) Utilization of distinct signaling pathways by receptors for vascular endothelial cell growth factor and other mitogens in the induction of endothelial cell proliferation. *J. Biol. Chem.* **275,** 5096–5103

30. Dimmeler, S., and Zeiher, A. M. (2000) Akt takes center stage in angiogenesis signaling. *Circ. Res.* **86,** 4–5

31. Risau, W. Mechanisms of angiogenesis. (1997) *Nature* **386,** 671–674

32. Ferrara, N., and Davis-Smyth, T. (1997) The biology of vascular endothelial growth factor. *Endocr. Rev.* **18,** 4–25

33. Papapetropoulos, A., Garcia-Cardena, G., Dengler, T. J., Maisonpierre, P. C., Yancopoulos, G. D., and Sessa, W. C. (1999) Direct actions of angiopoietin-1 on human endothelium: evidence for network stabilization, cell survival, and interaction with other angiogenic growth factors. *Lab. Invest.* **79,** 213–223

34. Klagsburn, M., and D'Amore, P. (1991) Regulators of angiogenesis. *Annu. Rev. Physiol.* **53,** 217–239

35. Schaller, M. D., and Parsons, J. T. (1994) Focal adhesion kinase and associated proteins. *Curr. Opin. Cell Biol.* **6,** 705–710

36. Abedi, H., and Zachary, I. (1995) Signaling mechanisms in the regulation of vascular cell migration. *Cardiovasc. Res.* **30,** 544–556

37. van der Geer, P., Hunter, T., and Lindberg, R. A. (1994) Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu. Rev. Cell Biol.* **10,** 251–337

38. Pepper, J. S., Montesano, R., Mandriota, S. J., Orci, L., and Vassalli, J. D. (1996) Angiogenesis: a paradigm for balanced extracellular proteolysis during cell migration and morphogenesis. *Enzyme Protein* **49,** 138–162

39. Grant, D. S., and Kleinman, H. K. (1997) Regulation of capillary formation by laminin and other components of the extracellular matrix. *EXS* **79,** 317–333

40. Frederick, J. Woessner, Jr. (1998) The matrix metalloproteinase family. In *Matrix Metalloproteinase* (Parks W.C., Mecham, R.P., eds.), pp. 1–14, Academic Press London

Received April 5, 2003; accepted June 19, 2003.

Fig. 1

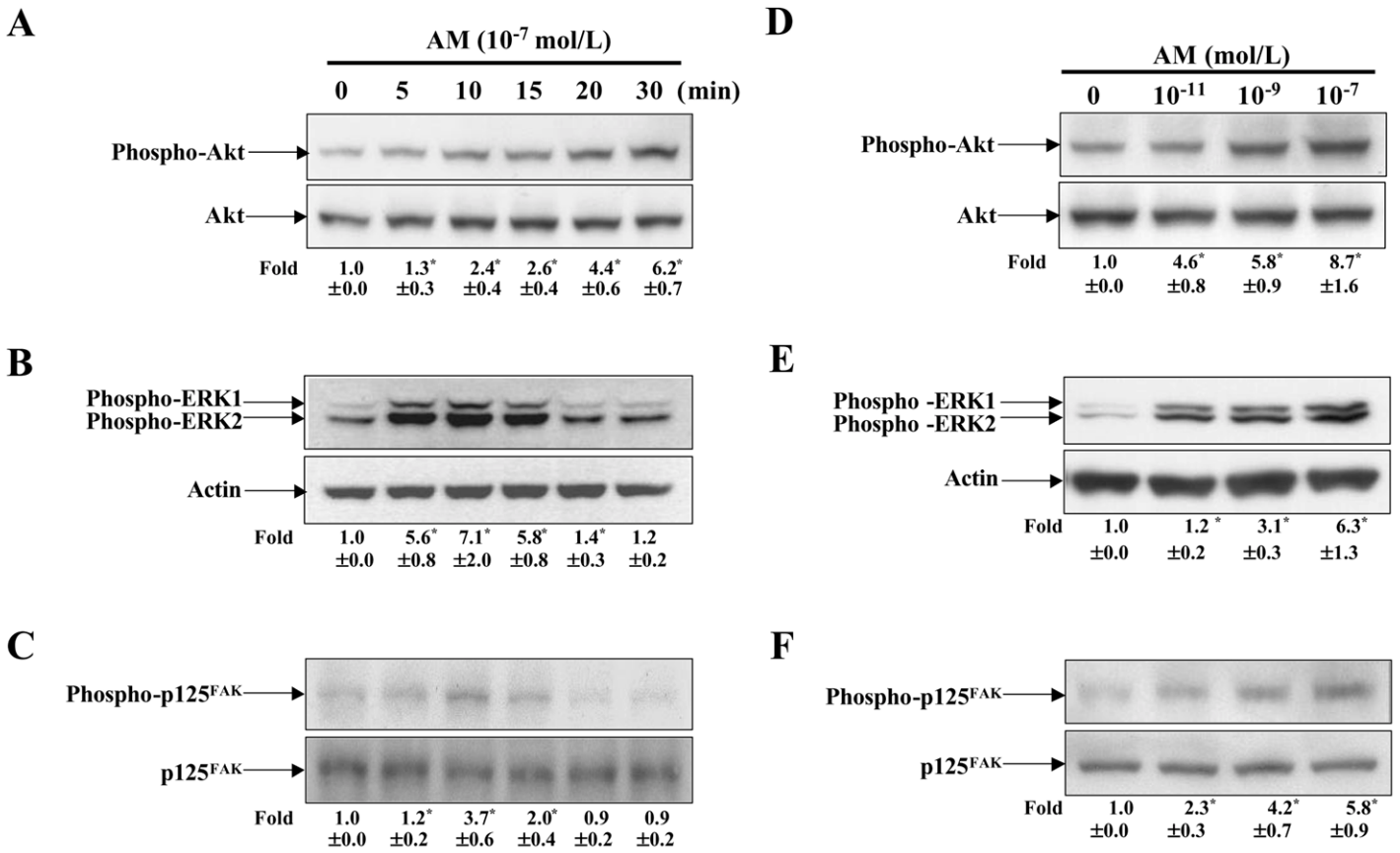

Figure 1. AM induces phosphorylation of Akt, ERK1/2, and p125^{FAK} in HUVECs. HUVECs were incubated for 16 h in 1% serum-containing M-199 medium, then incubated with AM (10^{-7} mol/l) for the indicated times (***A–C***) or incubated with varied concentrations of AM for 30 min (***D***) or 10 min (***E*** and ***F***). After treatment, cell lysates were harvested. Each lane contains 50 μ*g* of total protein from the cell lysates. Blots were probed with anti-phospho-Akt (***A*** and ***D***) or anti-phospho-ERK (***B*** and ***E***) or anti-phospho-p125^{FAK} antibody (***C*** and ***F***) (upper panels). The membrane was stripped and reprobed with anti-Akt antibody or anti-actin antibody or anti-p125^{FAK} antibody to verify equal loading of protein in each lane (lower panels). Fold: Densitometric analyses are presented as the relative ratio of phospho-Akt to Akt or phospho-ERK2 to actin, or phospho-p125^{FAK} to p125^{FAK}. The relative ratio measured at time 0 is arbitrarily presented as 1. Numbers represent the mean ±SD from three experiments. *P<0.05 vs. time 0 or control buffer.

Fig. 2

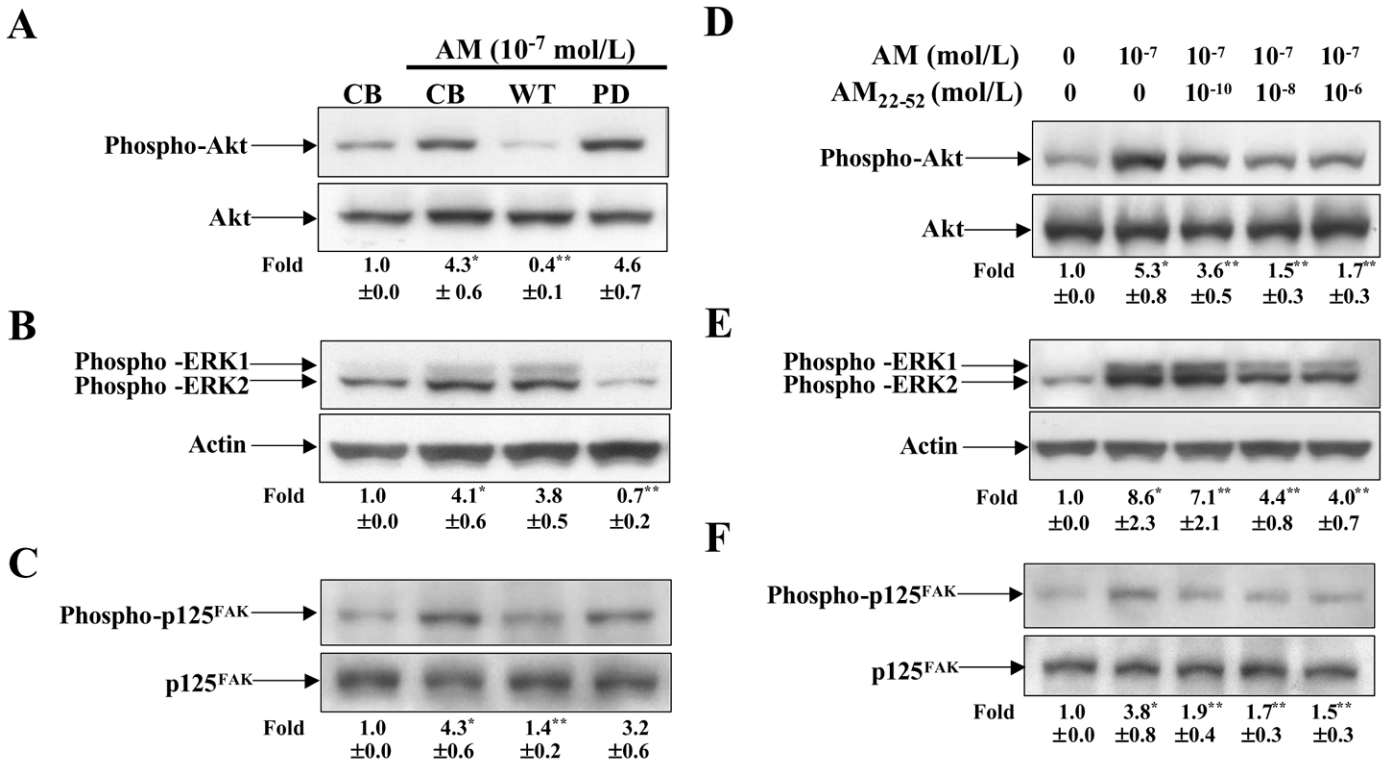

Figure 2. AM mediates phosphorylation of Akt and ERK using distinct signaling pathways and AM receptor blocker, AM_{22-52} partially inhibits AM-induced Akt, ERK1/2, and $p125^{FAK}$ phosphorylation. HUVECs were starved for 16 h in 1% serum-containing M199 medium. ***A–C)*** Cells were incubated in the presence of the indicated combinations of control buffer (CB), AM, wortmannin (WT, 30 nmol/l), or PD98059 (PD, 50 nmol/l) for 30 min (***A***) or 10 min (***B*** and ***C***). ***D–F)*** HUVECs were treated with AM and the indicated concentration of AM_{22-52} for 30 min (***D***) or 10 min (***E*** and ***F***). Blots were probed with anti-phospho-Akt (Ser473), anti-phospho-ERK, or anti-phospho-$p125^{FAK}$ antibody (upper panels). The membrane was stripped and reprobed with anti-Akt, anti-actin, or anti-$p125^{FAK}$ antibody to verify equal loading of protein in each lane (lower panels). Fold: Densitometric analyses are presented as the relative ratio of phospho-Akt to Akt, phospho-ERK2 to actin, or phospho-$p125^{FAK}$ to $p125^{FAK}$. The relative ratio measured at time 0, or the ratio relative to control buffer, is arbitrarily presented as 1. Numbers represent the mean ±SD from four experiments. **P*<0.05 vs. CB; ***P*<0.05 vs.CB+AM.

Fig. 3

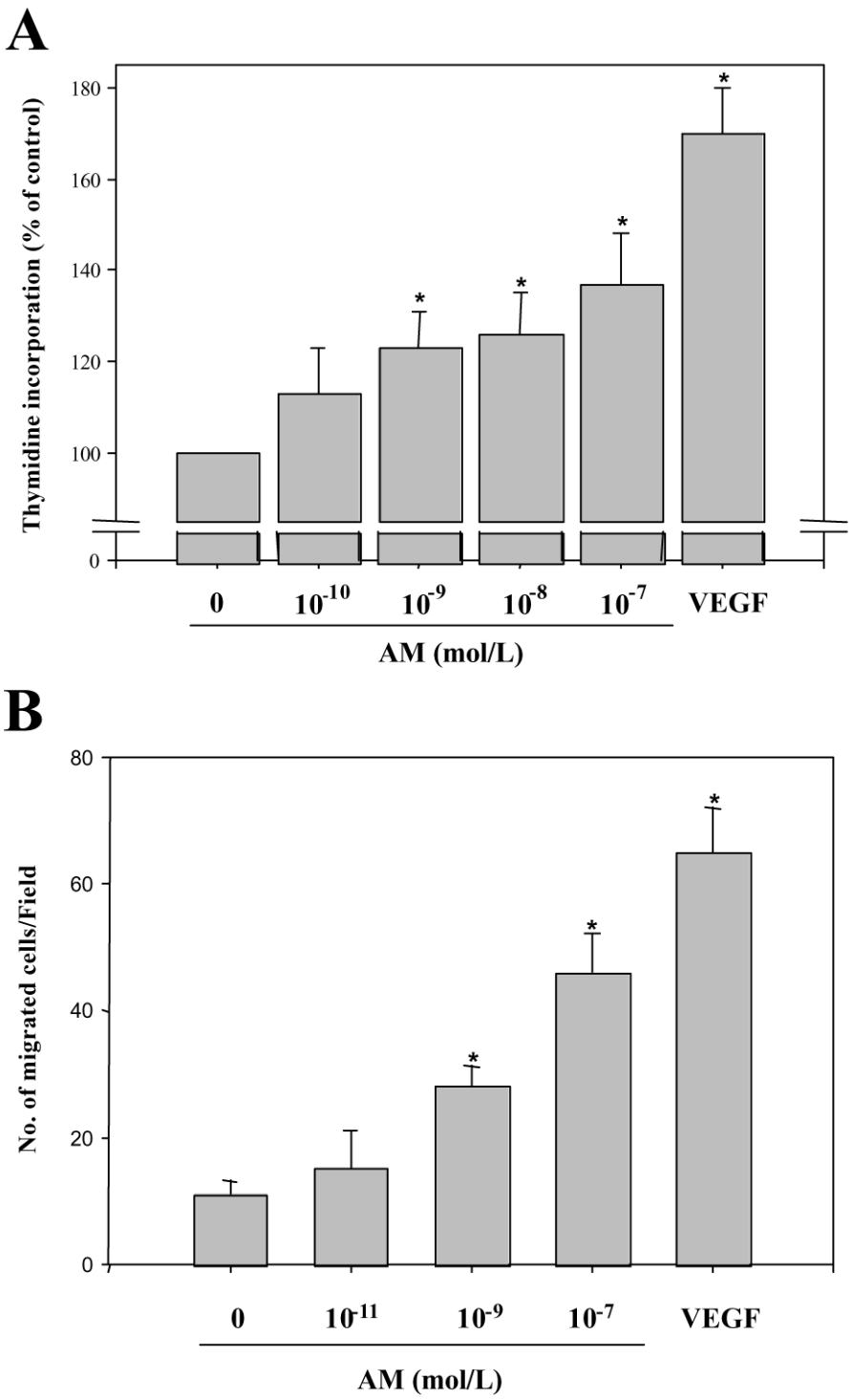

Figure 3. AM increases DNA synthesis and migration in endothelial cells. ***A)*** HUVECs were plated in M199 supplemented with 20% serum. After 18 h in culture medium with 1% FBS, control buffer (CB), AM (10^{-7} mol/l), or VEGF (10 ng/ml) was added in M199 medium supplemented with 2% serum. At 48 h, the DNA amount was measured by [^{3}H] thymidine incorporation assay. Thymidine incorporation in CB was arbitrarily set at 100. ***B)*** The bottom wells of the chamber were filled with M199 containing 1% bovine serum albumin and CB, AM (10^{-7} mol/l), or VEGF (10 ng/ml). HUVECs (2.0×10^{5}) were placed in the upper chamber, then incubated for 12 h at 37^{o}C. The migrated cells were stained with Diff-Quik solution and counted at 200× magnification using a microscope. Bars represent the mean ±SD from four experiments. *P<0.05 vs. CB.

Fig. 4

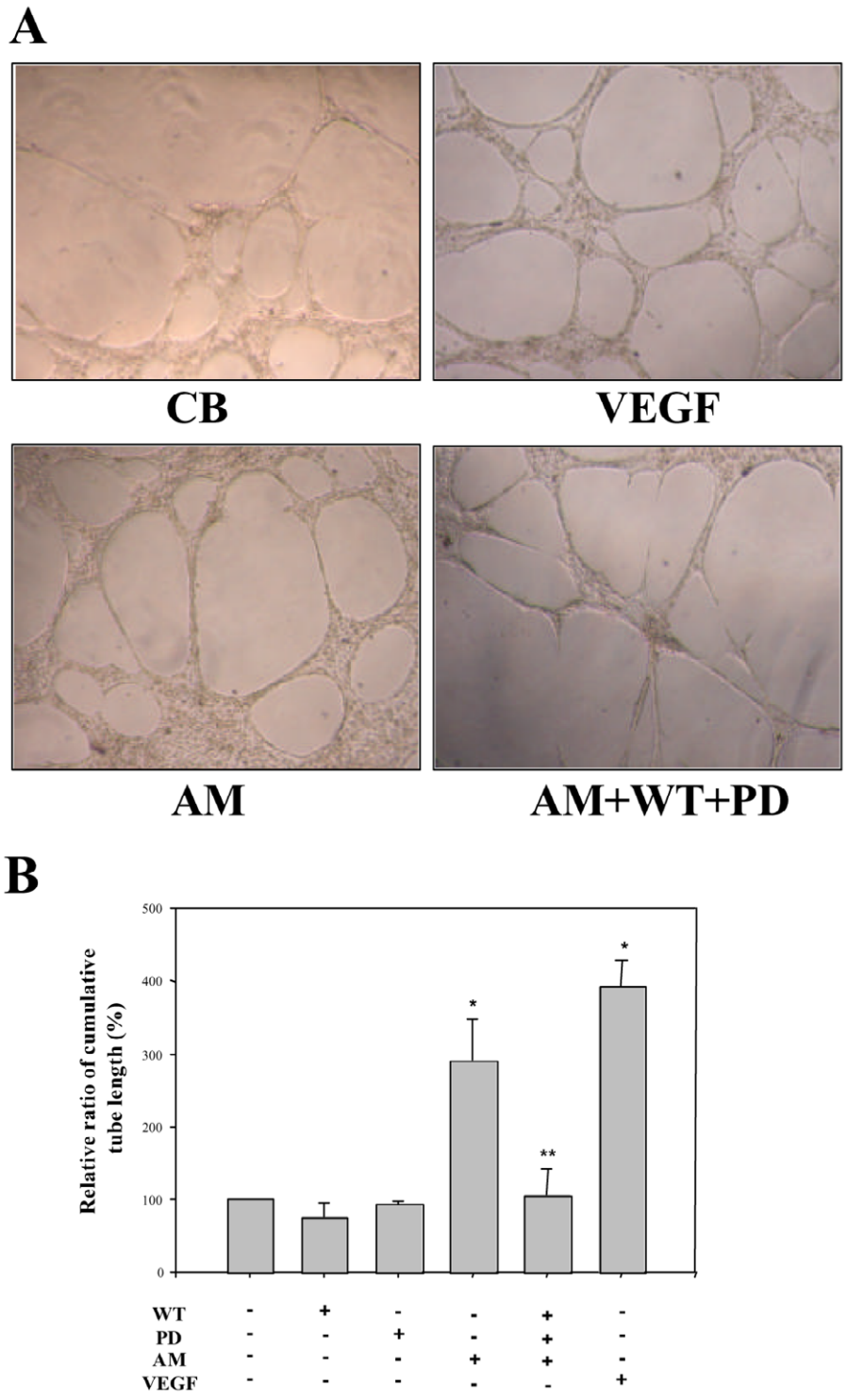

Figure 4. AM induces tube formation of endothelial cells in collagen gel. Gels were incubated for 48 h in the presence of the indicated combinations of control buffer (CB), AM (10^{-7} mol/l), PD98059 (PD, 2 μmol/l), wortmannin (WT, 30 nmol/l), or VEGF (10 ng/ml). Growth factors and inhibitors were replenished every 24 h. Endothelial tube formation was assayed in three-dimensional matrices with type I collagen gel. ***A)*** Representative phase-contrast photographs of capillary-like tube formation in collagen gel. Note that AM induced more tube formation than CB, but less than VEGF. AM-induced tube formation was suppressed by the addition of kinase inhibitors WT and PD. ***B)*** Quantification of tube formation. From phase contrast images, tube formation was quantified relative to the total length of tubes. Tube formation in the absence of AM is arbitrarily set at 100%. Bars represent the mean ±SD of three experiments. **P*<0.05 vs. CB; ***P*<0.05 vs. AM.

Fig. 5

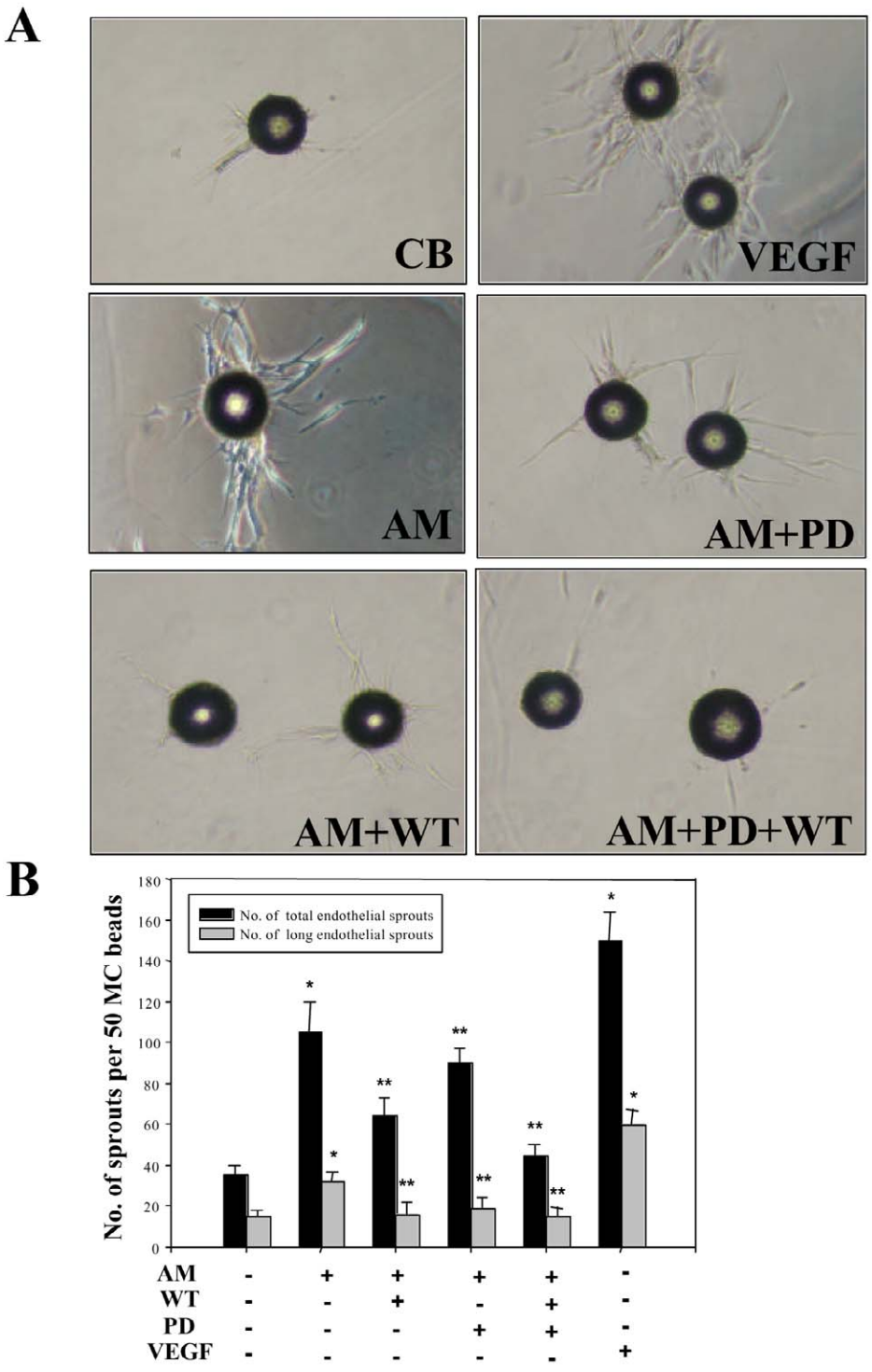

Figure 5. AM induces sprouting in PPAECs through PI 3'-kinase and the MEK1/2 pathway. Cells grown on microcarrier beads were placed in fibrin gels. Gels were incubated in the presence of the indicated combinations of: control buffer (CB), AM (10^{-7} mol/l), PD98059 (PD, 2 μmol/l), wortmannin (WT, 30 nmol/l), or VEGF (10 ng/ml). Growth factors and inhibitors were replenished every 24 h. ***A)*** Representative phase-contrast photographs of sprouting activity. Magnifications are ×200. ***B)*** Quantification of sprouting activity. The total number of endothelial sprouts and number of endothelial sprouts with length exceeding the diameter of the microcarrier beads (175 μm) per 50 microcarrier beads were counted after 2 days. Data are mean ±SD from five experiments. **P*<0.05 vs. control, ***P*<0.05 vs. AM.

Fig. 6

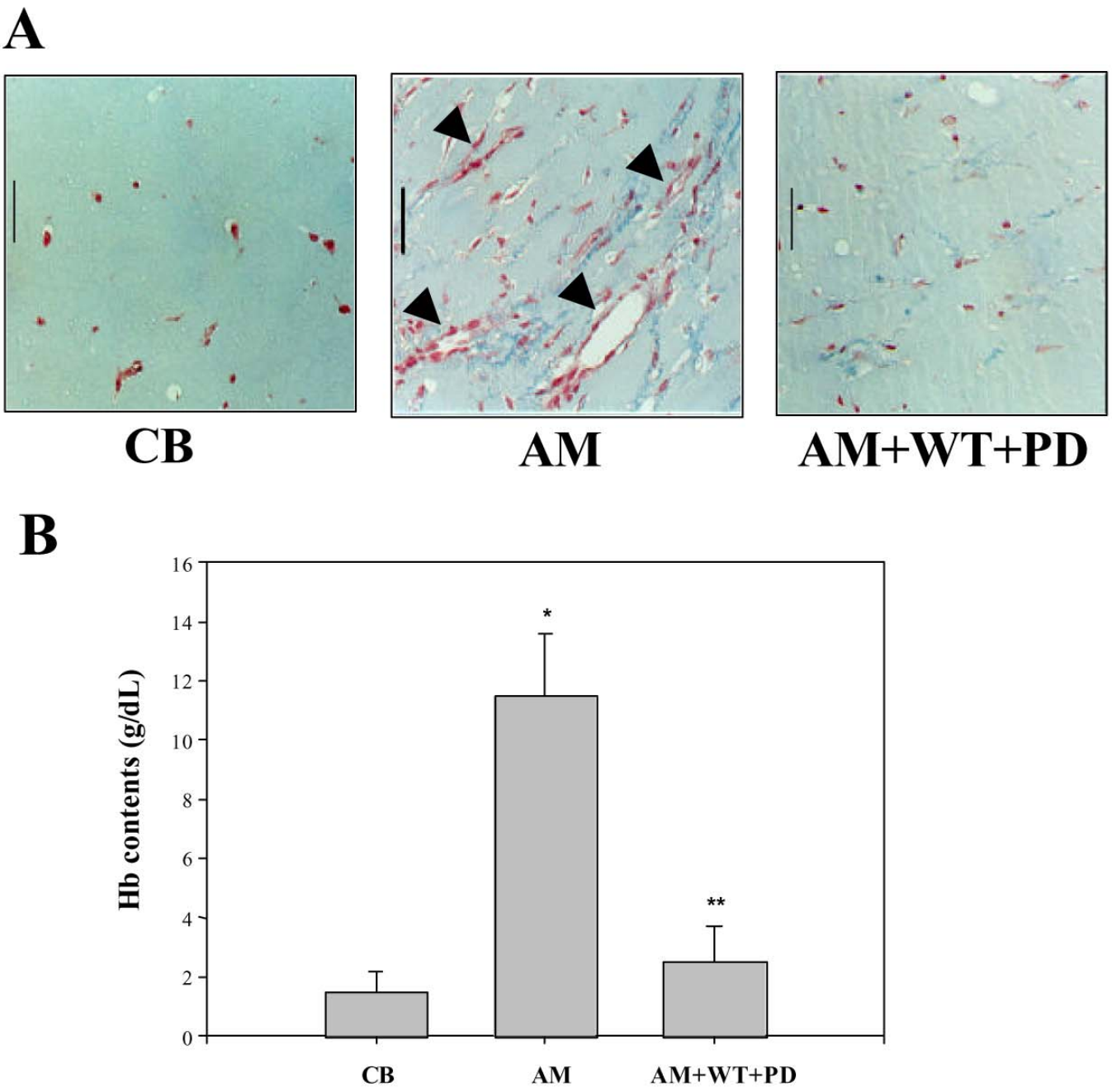

Figure 6. AM promotes neovessel formation in vivo in Matrigel plug. C57/BL6 mice were injected s.c. with 0.5 ml of Matrigel with control buffer or with AM (2 10^{-7} mol/l) with or without PD98059 (PD, 2 μmol/l) and wortmannin (WT, 30 nmol/l). After 2 wk, mice were killed and the Matrigel plugs were excised and fixed. ***A)*** Representative photograph of Matrigel plugs containing control buffer (CB), AM, or AM plus PD98059 and wortmannin (AM+WT+PD). Plugs were cross-sectioned and stained with Trichrome-Masson stain. Arrowheads indicate neovessels containing red blood cells. Bar, 50 μm. ***B)*** Quantification of neovessel formation by measurement of hemoglobin in the Matrigels. Six mice were used in each treatment group. *P<0.05 vs. CB; **P<0.05 vs. AM.

Fig. 7

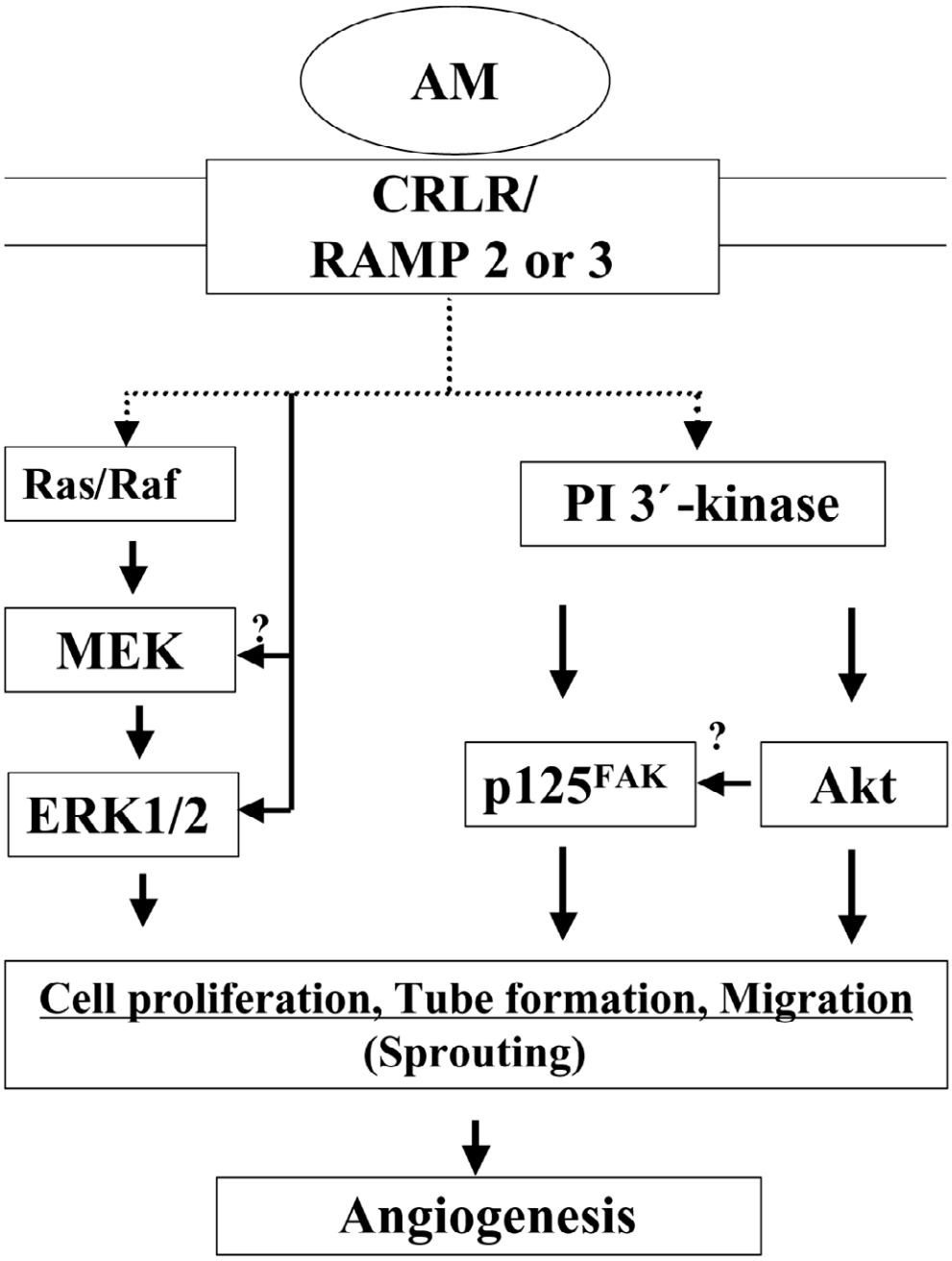

Figure 7. Model for AM-induced angiogenesis in endothelial cells. On the plasma membrane of the endothelial cell, AM binds to the CRLR/RAMP 2 or 3 functional receptor shown. Phosphatidylinositol 3'-kinase (PI 3'-kinase)/Akt, and mitogen-activated protein kinase (MAPK) pathways are activated. AM also activates focal adhesion kinase ($p125^{FAK}$). The downstream pathways are associated with endothelial proliferation, migration, and tube formation. In addition, AM induces sprouting in vitro. Taken together, these cellular processes promote angiogenesis in vivo.