Transactivation of KDR/Flk-1 by the B2 Receptor Induces Tube Formation in Human Coronary Endothelial Cells

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Abstract—Endothelial cells (ECs) are the critical cellular element responsible for postnatal angiogenesis. Vascular endothelial growth factor (VEGF) stimulates angiogenesis via the activation of kinase insert domain–containing receptor/fetal liver kinase-1 (KDR/Flk-1) in ECs. In addition, transactivation of KDR/Flk-1 by the bradykinin (BK) B2 receptor induces the activation of endothelial nitric oxide synthase (eNOS). These findings indicate that the precise role of BK in angiogenesis is likely to be more complex than initially thought, and it questions the importance of BK in angiogenic processes. Therefore, we examined whether transactivation by BK induced tube formation. We developed an in vitro model of human coronary artery EC (HCEC) tube formation on a matrix gel. We demonstrated that BK dose-dependently induced tube formation. Although a lower concentration of BK and VEGF did not separately induce tube formation, the formation was induced by a combination of lower concentrations of BK and VEGF, suggesting that VEGF and BK had a synergistic effect. The effect was blocked by a B2 receptor antagonist (HOE140) and specific inhibitors of VEGF receptor tyrosine kinases (Tki) and NOS. In addition, BK induced tyrosine phosphorylation of the KDR/Flk-1 receptor, as did VEGF itself. The transactivation was also blocked by HOE140 and Tki. Our results showed that, in HCECs, stimulation of the B2 receptor leads to the transactivation of KDR/Flk-1, as well as to eNOS activation, which induces tube formation. To our knowledge, this is a novel mechanism in which transactivation of KDR/Flk-1 by a G protein–coupled receptor, B2 receptor, may be a potent signal for tube formation. (Hypertension. 2003;41:1118-1123.)

Key Words: endothelium • endothelial growth factors • kinase • receptors, bradykinin • nitric oxide

Bradykinin (BK) is generated from the action of kallikreins on their substrate kininogen and acts via at least 2 BK receptor subtypes, B1 and B2, members of the G protein–coupled receptor (GPCR) family. B2 receptor is constitutively expressed in various tissues and is responsible for most of the effects of BK. In contrast, the B1 receptor has higher affinity for kinin metabolites, and its expression is induced under pathological conditions. BK signal transduction in endothelial cells (ECs) is mediated by the B2 receptor and involves the activation of phospholipases A2 and C. The activation of phospholipase C generates inositol triphosphate and diacylglycerol, causing intracellular Ca2+ mobilization and protein kinase C activation.

Because angiogenesis, the process of postnatal neovascularization, is a critical component of several human diseases, including ischemic heart disease, cancer, diabetic microvascular disease, rheumatoid arthritis, and psoriasis, we focused on the effect of BK on angiogenesis. Moreover, angiogenesis is believed to be mediated by the proliferation, migration, and remodeling of fully differentiated ECs.1,2 Several lines of evidence support the putative role of BK in the modulation of angiogenesis; eg, BK, in synergy with interleukin-1, enhances the angiogenic process in rat subcutaneous-sponge granuloma.3 Recently, local delivery of the tissue kallikrein gene has been shown to stimulate angiogenesis in both ischemic and normoperfused skeletal muscle through the activation of nitric oxide synthase and cyclooxygenase-2.4 The proangiogenic effect of angiotensin-converting enzyme (ACE) inhibition is mediated by B2 receptor signaling and is associated with the upregulation of endothelial nitric oxide synthase (eNOS) content, independent of vascular endothelial growth factor (VEGF) expression.5 While our experiments were in progress, Thuringer et al6 reported that transactivation of the VEGF receptor kinase insert domain–containing receptor/fetal liver kinase-1 (KDR/Flk-1) by BK contributed to eNOS activation in cardiac capillary ECs. These reports indicate that the precise role of BK in angiogenesis is likely to be more complex than initially thought, and it questions the importance of BK in angiogenic processes. Therefore, in an in vitro model of human coronary artery EC (HCEC) tube formation on a matrix gel, we showed that transactivation of KDR/Flk-1 by BK through the B2 receptor may be a potent signal for the angiogenic phenotype in HCECs.

Methods

Materials

The following antibodies and reagents were purchased: VEGF and L-NAME (N-o-nitro-L-arginine methyl ester hydrochloride) from Sigma; a specific inhibitor of VEGF receptor tyrosine kinases...
(4-[(4'-chloro-2'-fluoro)phenylaminol]-6,7-dimethoxy-quinazoline; Tki) (Calbiochem); HOE140 (D-arginyl-[Hyp 3, Thi 5, D-Tic 7, Oic 8]-BK) and BK from Peptide Institute Inc; a monoclonal anti-KDR/Flk-1 antibody (BD Biosciences); and a monoclonal anti-PY20 antibody (Upstate Biotechnology).

Cell Culture

HCECs were purchased from Clonetics. HCECs were cultured in EC growth medium (EGM, Clonetics) supplemented with 5% fetal bovine serum (FBS), penicillin/streptomycin, and EC growth supplement (Takara Co) at 37°C and 5% CO2.

Angiogenesis Assay on Matrix Gels

Matrix gels (Matrigel) were purchased from Chemicon International, Inc. The gels were allowed to polymerize in a 96-well plate for 1 hour at 37°C. HCECs were seeded at 1×10⁴ per well and grown in EGM supplemented with 0.1% FBS and without EC growth supplement for 18 hours in a humidified 37°C, 5% CO2 incubator. In some experiments, cells were cultured in the presence or absence of different kinds of reagents for 18 hours. After being washed, tube formation was observed with a light microscope, and pictures were captured with a computer system. In an attempt to automate the procedure, we performed a "pixel analysis" of the tube formation area. The image of the area was converted to a black scale. The image was subjected to image processing with NIH Image 1.62 software to calculate the total number of pixels. The number was counted in 3 different areas, and the average value was determined for each sample. The control sample was defined as 100% tube formation, and the percent increase or decrease in tube formation relative to the control was calculated for each sample (Figure 1).

Cell Proliferation Assay

HCECs (5×10⁴) were plated on a 96-well plate and cultured under 5% serum conditions. After 48 hours, the cells were cultured for 18 hours in the presence or absence of 10 to 0.0001 µmol/L BK in EGM supplemented with 0.1% FBS and without EC growth supplement at 37°C and 5% CO2. After 18 hours, the cells were stained (CellTiter 96 One Solution Reagent, a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS assay] (Promega) for 2 hours at 37°C and 5% CO2, and absorbance was recorded at 490 nm with a 96-well plate reader. In addition, cell number was also determined by using a hematocytometer. HCECs (5×10⁵) were plated on a 10-cm dish and cultured under 5% serum conditions. After 48 hours, the cells were cultured for 18 hours in the presence or absence of 0.1 µmol/L BK in EGM supplemented with 0.1% FBS and without EC growth supplement at 37°C and 5% CO2. After 18 hours, the cells were trypsinized and counted.

Apoptosis Assay

HCECs (5×10⁴) were grown on a 10-cm dish for 48 hours under 5% FBS and exposed to 0.01 µmol/L BK for 18 hours in EGM supplemented with 0.1% FBS and without EC growth supplement at 37°C and 5% CO2. Fluorescence-labeled annexin V (FITC–annexin V, Bender MedSystems) staining of HCECs was used as a functional index of early apoptosis. Quantitative analysis of percentage of stained cells was analyzed by flow cytometry.

Preparation of Protein Extract, Immunoblotting, and Immunoprecipitation

After 1 day in 0.1% FBS conditions, cell cultures were preconditioned with or without inhibitors or blockers for 30 minutes and
stimulated with the mediator for 10 minutes at 37°C and 5% CO₂. Cells were then scraped. The procedure for cell lysis and Western blot analysis of signaling proteins on membranes (Immobilon-P, Millipore Corp) has been described earlier. Horse-radish peroxidase–conjugated secondary antibody and an enhanced chemiluminescence system (Amersham) were used for detection, and the band intensity was quantified by digital image analysis. For the immunoprecipitation study, cells were lysed with cold immunoprecipitation buffer (1% Triton X-100, 150 mmol/L NaCl, 10 mmol/L Tris-HCl at pH 7.4, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.2 mmol/L sodium orthovanadate, 0.2 mmol/L phenylmethylsulfonyl fluoride, and 0.5% nonylphenol polyoxyethylene-40) with agitation for 30 minutes at 4°C. The cells were disrupted by brief sonication and centrifuged (16 000 g, 4°C, 15 minutes). The supernatant was mixed with primary antibody and incubated at 4°C for 1 hour. Protein A–agarose was then added to the supernatant, incubated at 4°C for 30 minutes at 4°C, and centrifuged, and the supernatant was removed. The pellet was washed twice with immunoprecipitation buffer, resuspended in electrophoresis sample buffer (250 mmol/L Tris-HCl at pH 6.8, 4% sodium dodecyl sulfate, 10% glycerol, 0.006% bromophenol blue, and 2% 2-mercaptoethanol), and analyzed. Densitometric analysis was performed in 3 independent experiments.

Statistical Analysis
Results are given as the mean±SEM. The significance of differences between mean values was evaluated by Student t test or 1-way ANOVA, as appropriate. A value of P<0.05 was considered significant.

Results
VEGF Promoted Tube Formation
We first examined the ability of VEGF to stimulate and stabilize tube formation by HCECs cultured on Matrigel. As shown in Figure 1, exposure of HCECs to VEGF led to the formation of capillary-like structures on the Matrigel surface; this proangiogenic effect was maximal after 8 hours, and we found that the optimal dose of VEGF for the maximum stimulation of tube formation was 50 ng/mL.

BK Stimulated Tube Formation
As shown in Figure 2, BK induced tube formation, and the dose response demonstrated that the maximum effective dose of BK was 0.1 μmol/L; this effect was completely blocked by HOE140, a B2 receptor antagonist. The angiogenic effect of ACE inhibition is mediated by B2 receptor signaling. Parenti et al⁸ reported that the B1 receptor promotes angiogenesis via the upregulation of endogenous fibroblast growth factor-2 (FGF-2) in the rabbit cornea. In our in vitro system, the B2 receptor, but not the B1 receptor, is expressed in HCECs based on reverse transcription–polymerase chain reaction (Miura et al, unpublished observations). Although this does not imply that the B1 receptor does not play a role in angiogenesis, BK induced angiogenesis through B2 receptor in our system.

BK Stimulated EC Proliferation Accompanied by Reduction in Apoptosis
Next, we examined the effect of BK on EC proliferation because growth stimulation induced 2 different steps, invasion and differentiation of ECs, in the angiogenic process. As shown in Figure 3A, BK induced cell proliferation by the MTS assay, and the dose response demonstrated that the maximum effective dose of BK was 0.1 μmol/L. Because the number of cells in the absence of 0.1 μmol/L BK decreased in contrast to the presence of it (Figure 3B), we measured the apoptotic effect under the same conditions (Figure 3C). Although only 2±2% of cells showed apoptosis in the presence of 0.1 μmol/L BK, 14±3% of apoptotic cells were seen in the absence of it. Therefore, 0.1 μmol/L BK stimu-
lated EC proliferation, accompanied by a reduction in apoptosis, and subsequently promoted tube formation.

**TKI and L-NAME Blocked BK-Induced Tube Formation**

Transactivation of the VEGF receptor KDR/Flk-1 by BK contributed to eNOS activation in cardiac capillary ECs. It is possible that this transactivation by BK through the B2 receptor induces tube formation. Therefore, we next examined the effect of TKI, a specific inhibitor of KDR/Flk-1 tyrosine kinases, on BK-induced tube formation and optimized the dose of the inhibitor to the minimum dose required to inhibit activation in initial experiments, where the dose response demonstrated that the minimum effective dose of TKI was 10 μmol/L (data not shown). As shown in Figure 4, inhibition of KDR/Flk-1 tyrosine kinase suppressed BK-induced tube formation.

We next sought to examine the role of eNOS activation in regulating tube formation. Experiments by He et al demonstrated that an NOS inhibitor, L-NAME, blocked VEGF-induced NO production through KDR/Flk-1 activation. In other experiments, VEGF was shown to be a potent EC-specific mitogen that promotes angiogenesis, vascular hyperpermeability, and vasodilation by NO. These data directly implicate NO as a critical modulator of angiogenesis. As shown in Figure 4, NOS inhibition blocked BK-induced tube formation.

**Transactivation of KDR/Flk-1 by BK Through the B2 Receptor**

KDR/Flk-1 is activated through ligand-stimulated receptor dimerization and the phosphorylation of tyrosine residues in the cytoplasmic kinase domain. Figure 5 shows that VEGF and BK induced the tyrosine phosphorylation of KDR/Flk-1. Immunoprecipitation by blotting with the anti-PY20 antibody revealed a dominant band (210 kDa) corresponding to the mature form of the phosphorylated KDR/Flk-1 receptor in BK- and VEGF-treated cells. The phosphorylation of KDR/Flk-1 peaked at 10 minutes, and at the maximum effective dose of BK for BK-induced transactivation (0.1 μmol/L; data not shown), this effect was completely blocked by HOE140, a B2 receptor antagonist. Transactivation of KDR/Flk-1 was mediated by B2 receptor signaling. We also examined the effect of TKI on BK-induced transactivation. TKI blocked not only the VEGF-induced phosphorylation of KDR/Flk-1 but also BK-induced phosphorylation.

**VEGF and BK Have a Synergistic Effect in Tube Formation**

Because B2 receptor signaling by BK occurs via transactivation, VEGF and BK should have either synergistic or additive effects in tube formation. To clarify these effects, we used lower concentrations of VEGF and BK, because neither 10 ng/mL VEGF nor 0.01 μmol/L BK induced tube formation. In contrast, 10 ng/mL VEGF combined with 0.01 μmol/L BK induced tube formation, and this effect was blocked by TKI, as shown in Figure 6. Therefore, VEGF and BK have a synergistic effect in inducing tube formation.
Discussion

Angiogenesis is a tightly regulated process that is integral to both normal and pathological conditions. In this study, we addressed the hypothesis that the contribution of BK to angiogenesis depends on the type of receptor transactivated in the endothelium and the ability to trigger an autocrine loop via the NOS pathway through the VEGF KDR/Flk-1 receptor.

Two subtypes of BK receptors, B1 and B2, have been defined based on their pharmacological properties. Most of the effects of BK have been reported to be linked to B2 receptor activation, whereas the functions of the B1 receptor are largely unknown. Regarding the mechanism of angiogenesis by BK, the proangiogenic effect of ACE inhibition is mediated by B2 receptor signaling and is associated with the upregulation of eNOS in the rabbit ischemic hindlimb. On the other hand, the angiogenic effect of BK is due to activation of the B1 receptor, which transduces the autocrine upregulation of FGF-2 by endogenous activation of the NOS pathway. In our in vitro system, it is important to note that the B1 receptor was not endogenously expressed in HCECs, and HOE140, a selective antagonist of B2 receptor, clearly blocked BK-induced angiogenesis. On the basis of these results, we propose a model for BK signaling through the B2 receptor, leading to angiogenesis.

Recent reports have implicated NO as a critical regulatory molecule for angiogenesis. BK mainly induces angiogenesis through activation of an NO signal pathway. Although activation of this angiogenic process was not associated with...

Figure 5. BK-induced transactivation of KDR/Flk-1 through B2 receptor. Representative pictures of immunoblotting. A, Control; B, VEGF 50 ng/mL; C, BK 0.1 μmol/L; D, VEGF 50 ng/mL+Tyk1 10 μmol/L; E, BK 0.1 μmol/L+Tyk1 10 μmol/L; and F, BK 0.1 μmol/L+HOE140 1 μmol/L. Graph shows intensities of bands compared with that in control by densitometric analysis (n=3, mean±SEM). *P<0.05 vs control.

Figure 6. VEGF and BK have synergistic effects on tube formation. Representative pictures of HCECs plated on Matrigel for 18 hours (A–G). A, Control; B, VEGF 10 ng/mL; C, VEGF 50 ng/mL; D, BK 0.01 μmol/L; E, VEGF 10 ng/mL+ BK 0.01 μmol/L; and G, VEGF 10 ng/mL+ BK 0.1 μmol/L+ Tyk1 10 μmol/L. Graph shows percent tube formation compared with that in control (n=3, mean±SEM). *P<0.05 vs control.
changes in VEGF protein levels, which suggests that this effect is independent of a VEGF pathway. VEGF is a potent, EC-specific mitogen that stimulates angiogenesis, vascular hyperpermeability, and vasodilation. It is unclear whether potentiation of the effect of BK affects the VEGF pathway. Three tyrosine kinase signaling receptors for VEGF have been identified: Flt-1, KDR/Flk-1, and Flt-4. The specific actions of VEGF on vascular ECs are regulated by Flt-1 and KDR/Flk-1. For instance, homozygous disruption of the KDR/Flk-1 gene leads to embryonic death due to failure of vasculogenesis, whereas homozygous Flt-1 disruption allows for normal vascular endothelial differentiation and development but leads to a failure to assemble normal vascular channels and death. KDR/Flk-1 is involved primarily in mitogenesis. Although VEGF production of NO through KDR/Flk-1 activation of c-Src, which leads to phospholipase C1 activation, inositol 1,4,5-triphosphate formation, and the release of [Ca\(^{2+}\)] has been established, the relation between BK and angiogenesis through KDR/Flk-1 is not well understood. Our results reflect the first comprehensive analysis of the role of BK-induced transactivation on this point. Our results are consistent with those of Thuringer et al, who reported that transactivation of KDR/Flk-1 by Flt-1 and KDR/Flk-1. For instance, homozygous disruption of the KDR/Flk-1 gene leads to embryonic death due to failure of vasculogenesis, whereas homozygous Flt-1 disruption allows for normal vascular endothelial differentiation and development but leads to a failure to assemble normal vascular channels and death. KDR/Flk-1 is involved primarily in mitogenesis. Although VEGF production of NO through KDR/Flk-1 activation of c-Src, which leads to phospholipase C1 activation, inositol 1,4,5-triphosphate formation, and the release of [Ca\(^{2+}\)], has been established, the relation between BK and angiogenesis through KDR/Flk-1 is not well understood. Our results reflect the first comprehensive analysis of the role of BK-induced transactivation on this point. Our results are consistent with those of Thuringer et al, who reported that transactivation of KDR/Flk-1 by Flt-1 and KDR/Flk-1. For instance, homozygous disruption of the KDR/Flk-1 gene leads to embryonic death due to failure of vasculogenesis, whereas homozygous Flt-1 disruption allows for normal vascular endothelial differentiation and development but leads to a failure to assemble normal vascular channels and death. KDR/Flk-1 is involved primarily in mitogenesis.

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