

Small Molecule Derived From Carboxyethylpyrrole Protein Adducts Promotes Angiogenesis in a Mouse Model of Peripheral Arterial Disease

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Background—CEP (ω -[2-carboxyethyl]pyrrole) protein adducts are the end products of lipid oxidation associated with inflammation and have been implicated in the induction of angiogenesis in pathological conditions such as tissue ischemia. We synthesized small molecules derived from CEP protein adducts and evaluated the angiogenic effect of the CEP analog CEP03 in the setting of peripheral arterial disease.

Methods and Results—The angiogenic effect of CEP03 was assessed by in vitro analysis of primary human microvascular endothelial cell proliferation and tubelike formation in Matrigel (Corning). In the presence of CEP03, proliferation of endothelial cells in vitro increased by $27 \pm 18\%$ under hypoxic (1% O_2) conditions, reaching similar levels to that of VEGFA (vascular endothelial growth factor A) stimulation ($22 \pm 10\%$), relative to the vehicle control treatment. A similar effect of CEP03 was demonstrated in the increased number of tubelike branches in Matrigel, reaching $>70\%$ induction in hypoxia, compared with the vehicle control. The therapeutic potential of CEP03 was further evaluated in a mouse model of peripheral arterial disease by quantification of blood perfusion recovery and capillary density. In the ischemic hind limb, treatment of CEP03 encapsulated within Matrigel significantly enhanced blood perfusion by 2-fold after 14 days compared with those treated with Matrigel alone. Moreover, these results concurred with histological finding that treatment of CEP03 in Matrigel resulted in a significant increase in microvessel density compared with Matrigel alone.

Conclusions—Our data suggest that CEP03 has a profound positive effect on angiogenesis and neovessel formation and thus has therapeutic potential for treatment of peripheral arterial disease. (*J Am Heart Assoc.* 2018;7:e009234. DOI: 10.1161/JAHA.118.009234.)

Key Words: angiogenesis • endothelial cell • peripheral vascular disease • small molecules

Peripheral arterial disease (PAD), an atherosclerotic occlusive cardiovascular disease of the peripheral arteries of the limb, is characterized by intermittent claudication, painful ischemic ulcerations, and even limb-threatening gangrene.¹ PAD is associated with dysfunction of or damage to vascular endothelium, a layer of endothelial cells (ECs) within blood vessels that play an important role in regulating vascular reactivity, remodeling, and angiogenesis. A

prominent treatment strategy is therapeutic angiogenesis, the sprouting of new blood vessels from preexisting vessels, which can lead to improved blood perfusion in the ischemic regions of the tissue. Biological approaches using angiogenic proteins, such as VEGFA (vascular endothelial growth factor A), bFGF (basic fibroblastic growth factor), and SDF-1 α (stromal cell-derived factor 1 α), have shown promising outcome in preclinical models but resulted in limited benefits

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Clinical Perspective

What Is New?

- We synthesized small molecules derived from CEP (ω -[2-carboxyethyl]pyrrole) protein adducts and evaluated the angiogenic effect of the CEP analog CEP03 in the setting of peripheral arterial disease.

What Are the Clinical Implications?

- Delivery of CEP03 may provide a therapeutic benefit for enhancing angiogenesis in patients with peripheral arterial disease.

in some clinical trials.^{2–4} Cell-based approaches using bone marrow mononuclear cells have been tested in a number of small randomized clinical trials, but the results are controversial because the improvement in ankle-brachial index was not consistent in some of the studies.^{5–7} Consequently, there is an increasing need for new angiogenic therapies.

As a kind of carboxyalkylpyrrole protein adduct, CEP (ω -[2-carboxyethyl]pyrrole) protein adducts are an end product of lipid oxidation that accumulate transiently during inflammation and wound healing.^{8,9} Although CEP protein adducts hold promise for developing proangiogenic therapeutics, similar concerns exist as with growth factors in drug development, including batch-to-batch variation, high cost of generating recombinant proteins, and a complex process to reach scalable quantities. Alternatively, small molecules might provide a new avenue to discover proangiogenic drugs.^{10,11} The advantages of small molecules include that they are chemically stable, easy to modify, and reproducibly manufactured in the cost-effective manner, and their effects are highly tunable and reversible.

In the light of the angiogenic effect of CEP protein adducts,⁸ we aimed to develop CEP-based small molecules that can promote neovascularization in ischemic limb tissues. By replacing the protein structure of CEP protein adducts with small chemical groups, we identified a new small molecule named CEP03 that has profound positive effects in angiogenesis *in vitro* by enhancing proliferation and tube formation of primary ECs under both normoxic and hypoxic conditions. When coinjected with Matrigel (Corning) to form plugs in subcutaneous pockets of mice, CEP03 significantly enhanced microvessel formation. More important, delivery of Matrigel containing CEP03 in a mouse model of hind limb ischemia not only significantly enhanced microvessel formation but also improved blood perfusion after 14 days. Consequently, these data suggest that CEP03 is a proangiogenic small molecule that may have therapeutic potential to enhance vascular formation in patients with PAD.

Material and Methods

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure. This material can be made available by the corresponding author on reasonable request.

Chemical Synthesis and Characterization

The CEP-based small molecules, such as CEP03, were synthesized in the laboratory from commercially available starting materials^{12,13} and reconstituted in DMSO (dimethyl sulfoxide). These small molecules were fully characterized by nuclear magnetic resonance spectroscopy and liquid chromatography–mass spectrometry: hydrogen 1 (¹H) nuclear magnetic resonance (400 MHz, CD₃OD) δ 9.92 (s, ¹H), 6.58 (m, ¹H), 5.95 (m, ¹H), 5.81 (m, ¹H), 2.86 (t, J=7.6 Hz, ²H), 2.58 (t, J=7.6 Hz, ²H); mass spectrometry (electrospray ionization): m/z 138.3 [M–H][–]. All CEPs tested in the assays have purity >95% based on proton (¹H) nuclear magnetic resonance and liquid chromatography–mass spectrometry. Compounds were dissolved in DMSO and were added at the indicated concentrations.

Cell Culture

Primary human dermal microvascular ECs were cultured in gelatin (0.2%) coated flasks in EGM-2MV (Lonza) growth medium. Primary rat endothelial progenitor cells (EPCs) were isolated from bone marrow, as published previously,^{14,15} and expanded for 14 days in EGM-2MV media before *in vitro* studies. Human EPCs were maintained and passaged in human EPC undifferentiation media with serum. Human fibroblasts (BJ, ATCC) were cultured in Dulbecco's modified Eagle's medium containing 10% FBS.

CEP Screening Test Based on Cell Proliferation

A total of 9 small molecule CEP derivatives were synthesized (CEP01–CEP09; chemical structures presented in Figure 1) and characterized for effect on EC proliferation via the CellTiter (Promega) assay. Primary ECs were seeded at a density of 1×10^4 cells/well in 96-well plates overnight in EGM-2MV medium under normoxic condition (21% O₂ and 5% CO₂). The ECs were then subjected to a 24-hour starvation period in EBM (Lonza) endothelial basal medium (phenol red free) supplemented with 0.2% FBS under hypoxic (1% O₂, 5% CO₂) conditions. On the following day, we prepared CEP compounds (–01 through –09) or VEGFA in sterile DMSO and then added them to starvation media at a final concentration of 5 μ mol/L for each CEP compound or

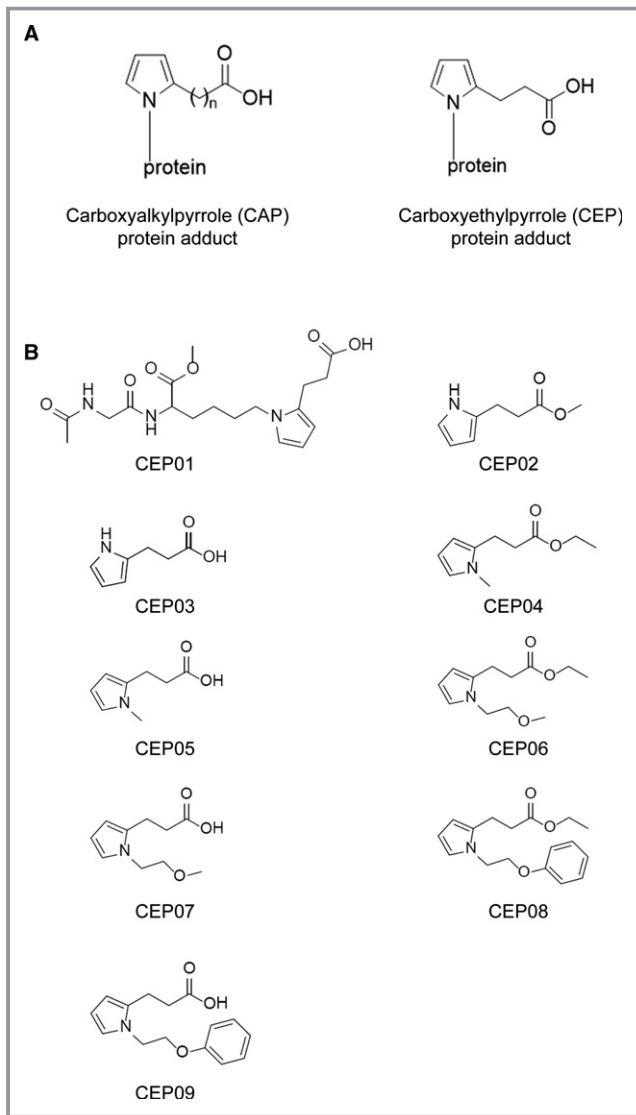


Figure 1. Carboxyethylpyrrole-related protein adducts and small molecules. A, Chemical structure of carboxyalkylpyrrole (CAP) protein adduct and CEP (ω -[2-carboxyethyl]pyrrole) adduct. B, Chemical structures of CEP derivative small molecules CEP01 through CEP09.

0.65 nmol/L for VEGFA. Sterile DMSO alone was added to media to serve as the vehicle control. Cell proliferation was quantitatively assessed after 24 hours of treatment by the CellTiter assay, according to manufacturer's instructions (Promega G3580), and quantified using a SoftMax Pro spectrophotometer at 490 nm wavelength (Figure 2A). To substantiate the positive effect of CEP03 on EC proliferation, the CellTiter assay was performed selectively in both normoxic and hypoxic conditions in the presence of CEP03 or VEGFA ($n \geq 6$). Cell proliferation was measured spectrophotometrically and compared with vehicle control and the VEGFA-treated group. Where specified, fibroblasts treated with VEGFA or CEP03 were assessed for proliferation using the same protocol as above.

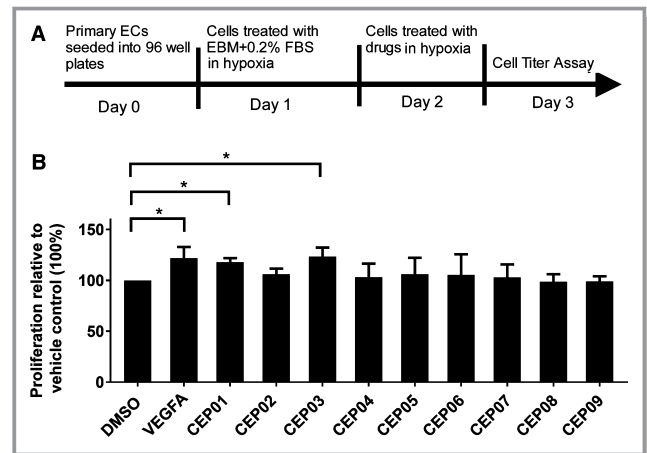


Figure 2. Carboxyethylpyrrole derivative small molecule CEP03 promotes primary endothelial cell (EC) proliferation under hypoxia conditions in vitro. A, Schematic diagram of the screening test method. B, CEP01 and CEP03 promote cell proliferation at a level comparable to VEGFA (vascular endothelial growth factor A) in human primary ECs under hypoxia (1% O_2). Cells were cultured in medium with dimethyl sulfoxide (DMSO, vehicle control), VEGFA (0.65 nmol/L), or CEP (ω -[2-carboxyethyl]pyrrole) small molecule derivatives CEP01 through CEP09 (5 μ mol/L) for 24 hours ($n=9$). * $P < 0.05$.

Because the TLR-2 (Toll-like receptor 2) signaling pathway has been shown to modulate the function of CEP-like small molecules,⁸ in some studies the ECs were incubated with TLR2 neutralizing antibody or isotype control before quantitative analysis of cell proliferation using the CellTiter assay, as described above ($n=3$).

Immunoblotting Assay

To interrogate the molecular basis of CEP03-induced angiogenesis, the activation of endothelial nitric oxide synthase (eNOS) in response to CEP03 treatment was investigated by immunoblotting. Primary ECs were cultured to confluency and then treated with 5 μ mol/L CEPs or 0.65 nmol/L VEGFA in EBM supplemented with 0.2% FBS under hypoxic (1% O_2 , 5% CO_2) conditions. At predetermined time points, cells from each sample were lysed in RIPA buffer supplemented with protease inhibitor cocktail. Equally loaded denatured protein lysates were separated by Invitrogen NuPAGE SDS-PAGE 4% to 12% Bis-Tris gels for 1 hour at 120 V and transferred to nitrocellulose membranes for 2 hours at 25 V. Membranes were blocked with 3% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 at room temperature before overnight incubation at 4°C with primary antibodies (phosphorylated eNOS or eNOS). After gentle washing with Tris-buffered saline containing 0.1% Tween-20, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Membranes were then reacted with Clarity Western substrate

for 5 minutes, and the resulting images were captured by the BioRad ChemiDoc XRS System for analysis.

Matrigel Tubelike Formation Assay

Human ECs were seeded at 1×10^5 cells per well onto 24-well plates coated with Matrigel (Geltrex [Gibco]; 300 μL /well) in starvation medium, which consisted of EBM supplemented with 1% FBS for 24 hours before the addition of vehicle control (DMSO), CEP03 (5 $\mu\text{mol/L}$), or VEGFA (0.65 nmol/L). Cells on Matrigel were incubated either at 37°C under normoxic or hypoxic conditions for 20 hours before phase-contrast images were captured using an inverted microscope. The number of branches were quantified by counting the number of branch points using ImageJ software (National Institutes of Health), as published previously,¹⁶ and normalized to the control (DMSO) group ($n \geq 6$). To confirm whether the effect of CEP03 is exclusive to mature ECs, rat EPCs were also tested under the same conditions ($n=9$).

EPC Wound-Healing Assay

Human EPCs were seeded in a Culture-Insert 2 well (Ibidi) with EGM-2MV medium at the cell density of 5×10^4 per well overnight, followed by a 24-hour starving period in 2% FBS medium. The Culture-Inserts were removed to create a uniform gap of 500 μm . Afterward, CEP03 (5 $\mu\text{mol/L}$), VEGFA (0.65 nmol/L), or DMSO vehicle control was added in EBM supplemented with 2% FBS ($n=3$). Cells with gaps were incubated in normoxic conditions for 24 hours before phase-contrast images were captured using an inverted microscope. The distances of gaps were measured, averaged, and normalized to the original condition using ImageJ and plotted as a percentage of closure using GraphPad Prism 7 (GraphPad Software). Similar studies were performed using primary rat EPCs isolated using established methods ($n=6$).¹⁷

Angiogenic Effect of CEP03 in a Subcutaneous Matrigel Plug Assay

The efficacy of CEPs was further tested *in vivo* in a murine subcutaneous Matrigel plug assay in C57/BL mice (aged 7–8 months, female). The animals were randomized to receive Matrigel alone (control), bFGF (6 nmol/L), CEP03 (2 mmol/L), or bFGF (6 nmol/L) plus CEP03 (2 mmol/L; $n=8$ per randomized group). Some groups were treated with bFGF as a reference control because bFGF is known to induce an angiogenic response in subcutaneous Matrigel plugs.¹⁸ All reagents were dissolved in Matrigel (Geltrex; Gibco) at 4°C and injected at 300 μL per injection subcutaneously to form a gelatinous plug. After 2 weeks, the animals were euthanized, and the plugs were harvested with the adjoining skin and

muscle intact, snap frozen in OCT compound for cryosectioning, and the immunofluorescently stained using a CD31 antibody for capillary density analysis. Capillary density for each animal was assessed by counting the number of the CD31-positive capillaries in 3 representative $\times 20$ images (BZ-X710 microscope; Keyence) in each of 4 tissue sections and expressed as number of capillaries per square millimeter.

Angiogenic Effect of CEP03 in the Ischemic Hind Limb

Hind limb ischemia was induced by unilateral ligation and excision of the femoral artery of 7- to 8-month-old female C57/BL mice, as published previously.^{19–21} The animals were randomly assigned to receive a 25- μL intramuscular injection into the gastrocnemius muscle of either Matrigel alone (control, $n=6$), or those supplemented with CEP03 (2 mmol/L) alone ($n=8$) or bFGF (6 nmol/L) plus CEP03 (2 mmol/L, $n=7$). Perfusion of ischemic hind limb was assessed using laser Doppler spectroscopy for up to 14 days. Laser Doppler images were quantitatively expressed as the mean perfusion ratio, which is the mean perfusion of the ischemic limb below the ankle divided by that of the contralateral unoperated limb below the ankle. At the end of the study period (14 days after surgery), the gastrocnemius tissues were harvested, snap frozen in OCT compound for cryosectioning, and stained using antibodies targeting CD31 or CD11b. Capillary density for each animal was assessed by counting the number of the CD31-positive capillaries in 3 representative $\times 20$ images (BZ-X710 microscope) in each of 4 tissue sections and expressed as number of capillaries per square millimeter. All animal studies were approved by the Administrative Panel on Laboratory Animal Care at Stanford University.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software and Primer of Biostatistics (v7). All data are expressed as mean \pm SD. For comparison between 2 groups, a Student *t* test was used. For comparison of ≥ 3 groups, 1-way ANOVA with Tukey adjustment was used. To evaluate the effect on each treatment group over time, repeated measures ANOVA with Tukey adjustment was used. Statistical significance was accepted at $P < 0.05$.

Results

CEP03 Promotes Endothelial Proliferation Under Hypoxia *In Vitro*

In an initial study, we synthesized 9 CEP small molecule entities and then screened these compounds for induction of

cell proliferation using primary human microvascular ECs. Among the 9 CEP analogs, CEP01 contains a dipeptide group that has been found to promote angiogenesis,⁸ but the remaining ones were not tested in the previous angiogenic assays. Based on our screen test for CEP01 through CEP09, we identified CEP03 (Figure 2B) as a chemical entity with a mitogenic effect similar to that of VEGFA. CEP03 (139 Da) is structurally similar to CEP01 but replaces the bulky dipeptide chain with a single hydrogen (Figure 1). ECs treated with CEP03 for 24 hours under hypoxia demonstrated $23.6\pm 8.8\%$ increases in proliferation compared with the vehicle control group.

To further substantiate the angiogenic effect of CEP03, we expanded our studies to evaluate cell proliferation at under either normoxic (21% O₂) or hypoxic (1% O₂) conditions, in which cells were treated with CEP01, CEP03, or VEGFA in EBM supplemented with 1% FBS for 24 hours. CEP03 significantly increased EC proliferation by $27\pm 19\%$ in normoxic condition and by $27\pm 18\%$ in hypoxic conditions ($P<0.05$). These relative increases were comparable to those caused by VEGFA^{22–24} ($32\pm 19\%$ and $22\pm 10\%$ under normoxic and hypoxic conditions, respectively; $P<0.05$; Figure 3). In addition, the enhancement in proliferation was not observed when CEP03 was incubated with fibroblast under the same conditions (Figure 4).⁸ These results demonstrate that CEP03 induced significant EC proliferation, suggesting that the heavy peptide arm on CEP01 is dispensable without diminishing promotion of cell proliferation.

CEP03 Enhances Endothelial Tubelike Formation Under Normoxia and Hypoxia In Vitro

CEP03 was next tested for proangiogenic capability using a Matrigel tubelike formation assay under normoxic (Figure 5A and 5B) or hypoxic (Figure 5C and 5D) conditions. Under normoxic conditions, ECs treated with CEP03 ($173\pm 35\%$) for 20 hours showed significantly more tubelike branches relative to the vehicle control (100%; $P<0.05$). The $\approx 70\%$ increase in tubelike structure formation associated with CEP03 treatment was comparable to the inductive effect of VEGFA in promoting endothelial tubelike formation in normoxia ($147\pm 26\%$). The similar probranching effect of CEP03 was observed under hypoxic conditions (CEP03: $177\pm 39\%$; VEGFA: $153\pm 28\%$). These results demonstrate that the CEP03 induced endothelial tubelike formation in Matrigel to a degree similar to VEGFA.

CEP03 Enhanced Migration of EPCs In Vitro

Besides assessing the angiogenic function of primary human ECs in the presence of CEP analogs, we also examined the angiogenic effects of CEP03 on human ECs in the progenitor

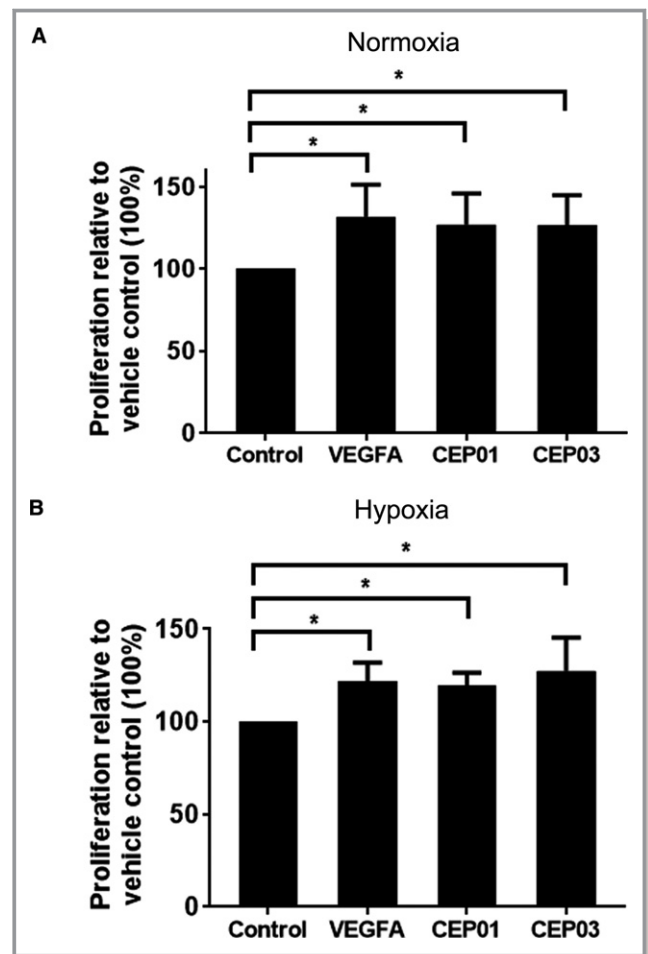


Figure 3. Carboxyethylpyrrole derivative small molecule CEP03 shows a pro-proliferation effect on primary endothelial cells (ECs) in both normoxic and hypoxic conditions. A and B, CEP01 and CEP03 promote cell proliferation at a level comparable to VEGFA in human primary ECs under both normoxic (A) and hypoxic (B) conditions. Cells were cultured in medium with dimethyl sulfoxide (vehicle control), VEGFA (vascular endothelial growth factor A; 0.65 nmol/L), or CEP01 or CEP03 (5 μ mol/L) for 24 hours ($n=9$). *Denotes significant comparison ($P<0.05$). CEP indicates ω -(2-carboxyethyl)pyrrole.

state. The migratory capacity of primary human EPCs in the presence of CEP03 was assessed in a wound-healing migration assay. In this assay, a defined gap was created within a confluent monolayer of EPCs, and the cells were then treated with CEP03, VEGFA, or vehicle control. After 24 hours, the migration of human EPCs was expressed as a percentage of the area of infiltration into the gap. As shown in Figure 6, the human EPCs treated with CEP03 migrated by a significantly greater distance into the center of the gap area under both normoxia ($61\pm 5\%$) and hypoxia ($43\pm 7\%$) compared with the vehicle control (normoxia: $34\pm 8\%$; hypoxia: $23\pm 1\%$), indicating an accelerated wound closure ($P<0.05$). Furthermore, the degree of migration in response to CEP03 was similar to that of VEGFA under normoxia ($66\pm 2\%$) and

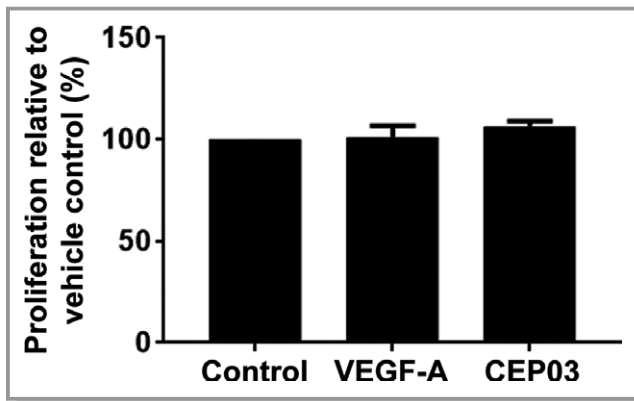


Figure 4. Carboxyethylpyrrole derivative small molecule CEP03 shows negligible pro-proliferative effect on other cell types such as fibroblasts. The enhancement in proliferation was not observed when CEP03 was applied to fibroblasts. Cells were cultured in medium with dimethyl sulfoxide (vehicle control), VEGFA (vascular endothelial growth factor A; 0.65 nmol/L), or CEP03 (5 μ mol/L) for 24 hours (n=9). CEP indicates ω -(2-carboxyethyl)pyrrole.

hypoxia ($36 \pm 6\%$). Similarly, as shown in Figure 7B, CEP03 also increased wound healing significantly in rat EPCs (CEP03: $40 \pm 14\%$; vehicle control: $17 \pm 6\%$; $P < 0.05$), along with a significant increase in tubelike formation (Figure 7A). Together, the in vitro assessment of proliferation, tubelike formation, and wound healing suggests the proangiogenic qualities of CEP03.

CEP03 Modulates EC Angiogenic Function by the eNOS Signaling Pathway

To better understand the mechanism by which CEP03 modulates EC function, we tested the involvement of TLR2, which has previously been shown to modulate some CEP-like small molecules.⁸ In the presence of TLR2 neutralization antibody, the proliferation of ECs treated with CEP03 showed no significant difference in comparison to cells treated with isotype control antibody (Figure 8), suggesting a TLR2-independent pathway. In contrast, immunoblotting analysis suggests that CEP03 acts, in part, through eNOS, as phosphorylation of eNOS could be observed within 1 hour of CEP03 treatment, similar to eNOS phosphorylation in response to the treatment of VEGFA (Figure 9). These studies suggest that eNOS may be an important modulator of CEP03 activity in ECs.

CEP03 Enhances Microvessel Formation in Subcutaneous Matrigel Plugs

Because CEP03 was able to significantly induce proliferation and tube formation in vitro, we next validated the angiogenic effects of CEP03 in vivo. Matrigel plugs were

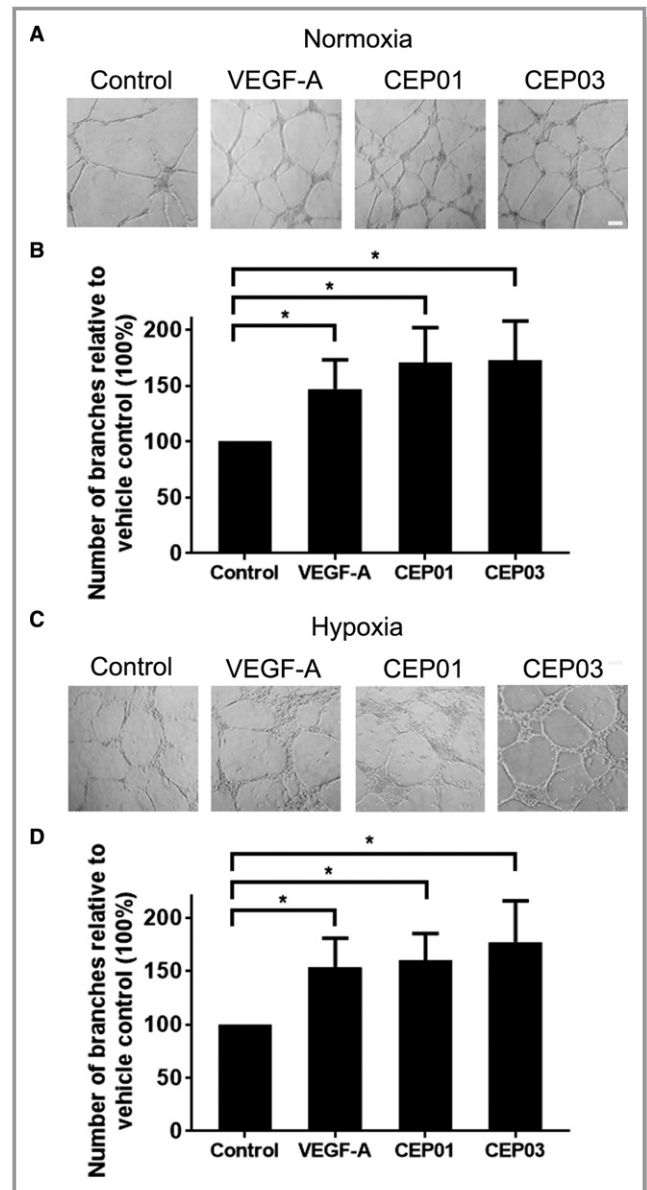


Figure 5. Carboxyethylpyrrole derivative small molecule CEP03 enhances tubelike formation under normoxia and hypoxia in vitro after 20 hours. A, Bright field images of tubelike structures formed on Matrigel in normoxia. Primary human endothelial cells were cultured in medium with dimethyl sulfoxide (vehicle control), VEGFA (vascular endothelial growth factor A; 0.65 nmol/L), CEP01 (5 μ mol/L), or CEP03 (5 μ mol/L). B, Quantification of branch numbers showed that VEGFA and CEP03 increased tube formation significantly ($n \geq 6$). C, Bright field images of tubelike structures formed on Matrigel under hypoxia (1% O_2). D, Quantification of branch numbers showed that VEGFA, CEP01, and CEP03 significantly increased tubelike formation under hypoxia (n=8). Scale bar=100 μ m. * $P < 0.05$. CEP indicates ω -(2-carboxyethyl)pyrrole.

generated in the abdominal subcutaneous space of C57/BL mice. To localize CEP03 to the subcutaneous space, a 2-mmol/L bolus of CEP03 was encapsulated within 300 μ L Matrigel and then coinjected. After 14 days, the

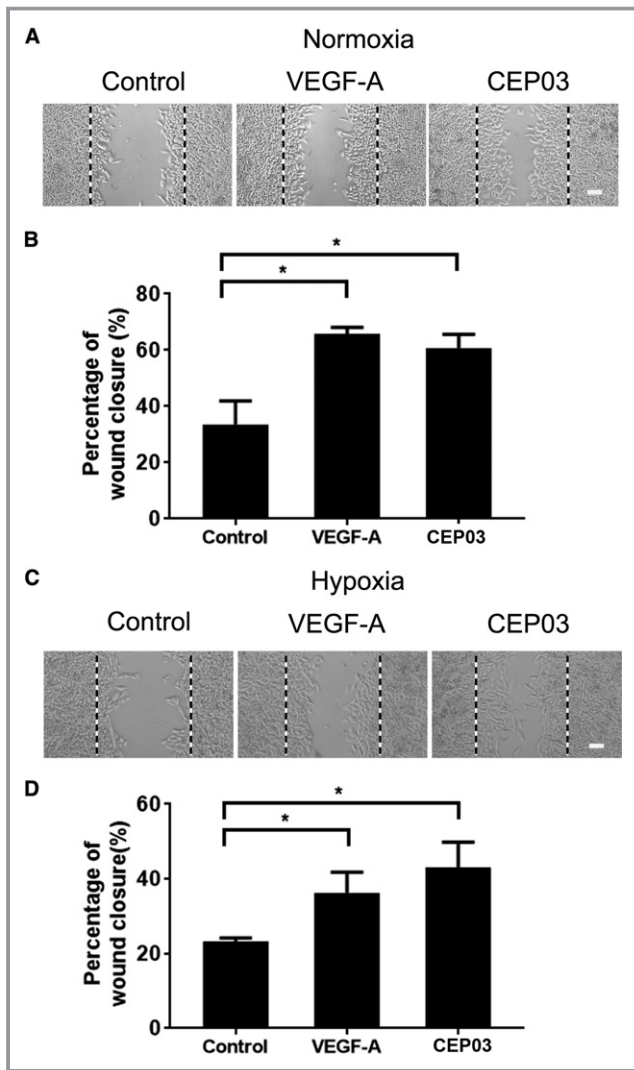


Figure 6. Carboxyethylpyrrole derivative small molecule CEP03 promotes wound healing in human endothelial progenitor cells (EPCs). A, Representative bright field images of wound healing experiments after 24 hours under normoxia. Human EPCs were cultured in medium with dimethyl sulfoxide (DMSO; vehicle control), VEGFA (vascular endothelial growth factor A; 0.65 nmol/L), or CEP03 (5 μ mol/L). B, Quantification of wound closure showed that VEGFA and CEP03 increased wound healing significantly compared with control (n=3). C, Representative bright field images of wound healing experiments after 24 hours under hypoxia. Human EPCs were cultured in medium with DMSO (control), VEGFA (0.65 nmol/L), or CEP03 (5 μ mol/L). D, Quantification of wound closure showed that VEGFA and CEP03 increased wound healing significantly compared with control (n=3). Scale bar=100 μ m. * P <0.05. CEP indicates ω -(2-carboxyethyl)pyrrole.

subcutaneous plugs were explanted, and immunostaining was performed using antibody against murine CD31 for identifying the microvessels. Some groups were treated with bFGF as a reference control because bFGF is known to induce an angiogenic response in subcutaneous Matrigel plugs.¹⁸ As shown in Figure 10, microvessel density was significantly

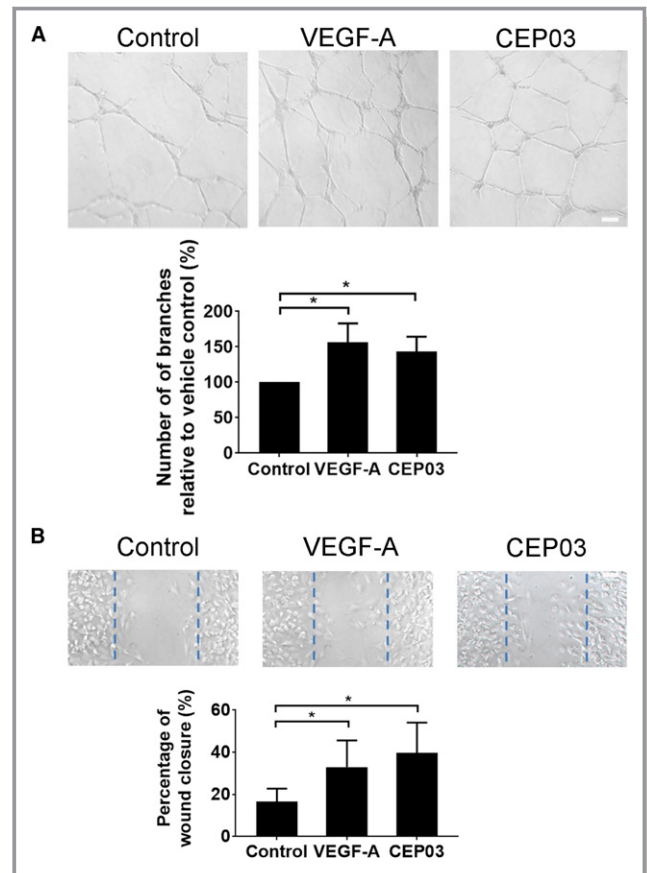


Figure 7. Carboxyethylpyrrole derivative small molecule CEP03 promotes tube formation and wound healing in rat endothelial progenitor cells under normoxia. A, CEP03 increases the number of tube branches compared with control (n=9). B, CEP03 markedly improves wound healing, comparable to that caused by VEGFA (vascular endothelial growth factor A; 0.65 nmol/L) treatment (n=6). Dotted line denotes the gap at 0 hour. Scale bar=100 μ m. *Significant comparison (P <0.05). CEP indicates ω -(2-carboxyethyl)pyrrole.

increased by the treatment of CEP03 ($27.6 \pm 14/\text{mm}^2$) or bFGF ($27.8 \pm 18.5/\text{mm}^2$) compared with vehicle control mice that received only Matrigel ($4.7 \pm 2.5/\text{mm}^2$; P <0.05). More important, when CEP03 and bFGF were delivered together within Matrigel, the increase in microvessel density ($76.1 \pm 23.8/\text{mm}^2$) was markedly higher, resulting in >16-fold higher capillary density compared with the control group receiving Matrigel plugs alone. These results demonstrate that CEP03 significantly induces microvessel formation in a subcutaneous plug, which concurs with our in vitro findings of increased angiogenesis under normoxic conditions.

CEP03 Enhanced Angiogenic Response in a Mouse Model of PAD

After demonstrating an angiogenic effect of CEP03 under physiological conditions, we further evaluated the therapeutic

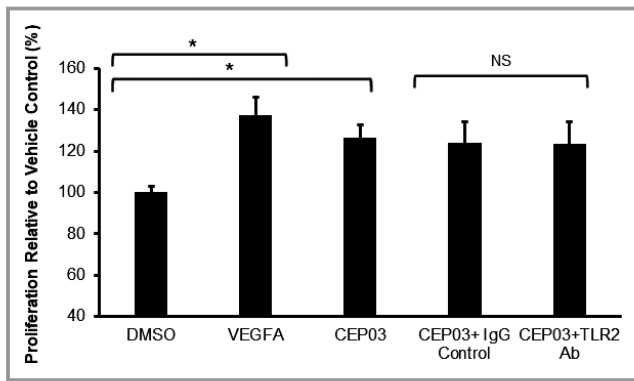


Figure 8. The angiogenic effect of carboxyethylpyrrole derivative small molecule CEP03 is not mediated by TLR2 (Toll-like receptor 2). In the presence of neutralization antibody (Ab) targeting TLR2, the proliferation of CEP03-treated endothelial cells is not significantly different compared with isotype control treatment ($n=3$). *Significant comparison ($P<0.05$). CEP indicates ω -(2-carboxyethyl)pyrrole; DMSO, dimethyl sulfoxide; NS, not significant.

efficacy of CEP03 in a preclinical mouse model of PAD by inducing unilateral hind limb ischemia. This disease model consists of ligating and excising the femoral artery, which leads instantaneously to a reduction in blood perfusion in the hind leg, as we demonstrated previously.²⁵ We tested the efficacy of CEP03 injected intramuscularly into the gastrocnemius muscle of 7- to 8-month-old C57/BL mice with induced hind limb ischemia. A 2-mmol/L bolus of CEP03 was encapsulated within Matrigel before injection into the ischemic hind limb. Over the course of 14 days, the mean perfusion ratio of the foot based on laser Doppler spectroscopy was assessed. As shown in Figure 11, the CEP03-treated animals showed a significant increase in relative mean perfusion ratio (ischemic/unoperated leg) after 14 days (0.6 ± 0.2) compared with Matrigel treatment alone (0.3 ± 0.1 ; $P<0.05$), although the combined delivery of CEP03 plus bFGF did not result in any further increase in blood perfusion (0.5 ± 0.1) compared with

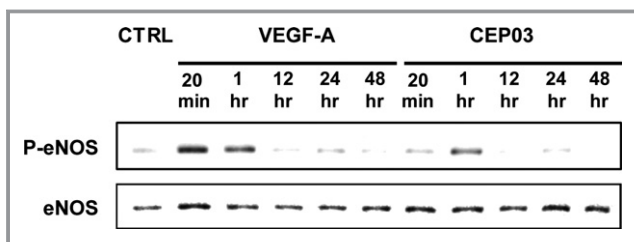


Figure 9. Activation of endothelial nitric oxide synthase (eNOS) in response to treatment of carboxyethylpyrrole derivative small molecule CEP03. Representative immunoblots demonstrate that CEP03 activates eNOS signaling, as does VEGFA (vascular endothelial growth factor A). CEP indicates ω -(2-carboxyethyl) pyrrole; p-eNOS, phosphorylated endothelial nitric oxide synthase.

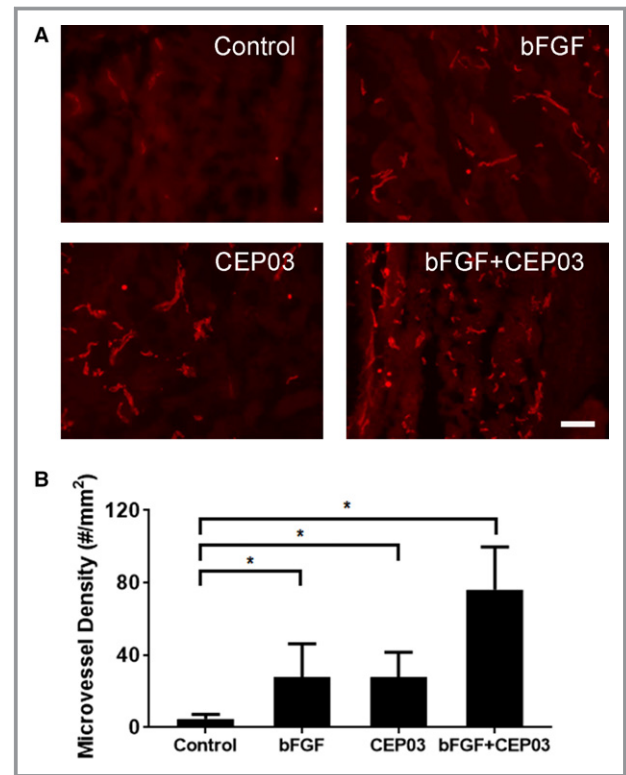


Figure 10. Carboxyethylpyrrole derivative small molecule CEP03 increases microvessel formation in subcutaneous Matrigel plugs in mice. A, Representative immunofluorescence images of Matrigel plug transverse sections after 14 days of transplantation show increased microvessels in Matrigel encapsulated with bFGF (basic fibroblast growth factor, CEP03, or bFGF plus CEP03). Red: CD31. B, Quantification of microvessel density show that bFGF, CEP03, and bFGF plus CEP03 increased microvessel formation significantly compared with the control ($n=8$ per randomized group). Scale bar=100 μ m. * $P<0.05$. CEP indicates ω -(2-carboxyethyl)pyrrole.

CEP03 delivery alone. As shown in Figure 12, when tissue sections of the ischemic gastrocnemius were immunofluorescently stained for CD31 to visualize microvessels, a significant increase in microvessel density in CEP03-treated ischemic limbs ($877\pm 83/\text{mm}^2$) was observed compared with the control mice ($721\pm 109/\text{mm}^2$). In addition, codelivery of bFGF and CEP03 within Matrigel led to a significant increase in mean microvessel density ($965\pm 137/\text{mm}^2$) compared with the control group but was not statistically different from that of the CEP03 treatment group. Immunofluorescence staining of pan macrophage marker CD11b revealed a negligible difference in macrophage infiltration among groups (Figure 13), indicating that the proangiogenic effect of CEP03 is independent of the inflammatory response in host tissue. Together, these in vivo studies demonstrate that CEP03 induces angiogenesis and blood flow recovery in the ischemic hind limb.

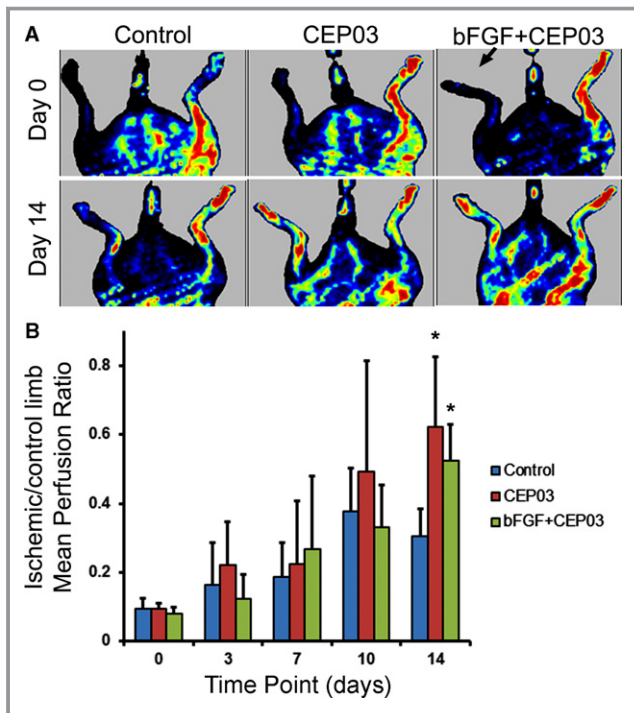


Figure 11. Carboxyethylpyrrole derivative small molecule CEP03 enhances limb perfusion after induction of hind limb ischemia. A, Representative laser Doppler images show recovery of perfusion in the ischemic limb (denoted by arrow) 14 days after surgery. B, Quantification of mean perfusion ratio (ischemic/unoperated limb) shows that treatment with CEP03 alone (n=8) or the combined treatment of CEP03 in conjunction with bFGF (basic fibroblast growth factor; CEP03+bFGF, n=7) enhanced blood perfusion 14 days after surgery relative to the control (n=6). * $P < 0.05$. CEP indicates ω -(2-carboxyethyl)pyrrole.

Discussion

In this work, we report the angiogenic effect of the small molecule CEP03, both in vitro and in vivo. The salient findings are (1) that CEP03 enhanced primary EC proliferation to a level comparable to that of VEGFA under normoxic and hypoxic conditions; (2) that CEP03 promoted endothelial tubelike structure formation of primary ECs under both normoxia and hypoxia; (3) that the in vitro angiogenic effects observed in ECs were recapitulated in EPCs based on the ability of CEP03 to significantly increase cell migration and tubelike formation; (4) that CEP03 promoted microvessel formation in subcutaneous Matrigel plugs in mice; and (5) that in a murine model of PAD, CEP03 significantly increased blood perfusion and microvessel density after 14 days. Based on these findings, CEP03 is a promising proangiogenic reagent that may improve the therapeutic effects under ischemic conditions such as heart failure or critical limb ischemia.

Traditional large peptide growth factors have encountered a number of limitations toward clinical translation, including high costs associated with recovery and purification,

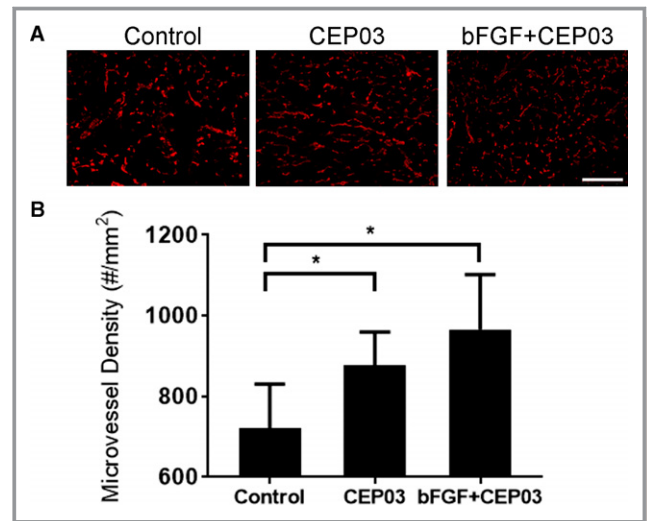


Figure 12. Carboxyethylpyrrole derivative small molecule CEP03 promotes microvessel formation in a mouse hind limb ischemia model. A, Representative immunofluorescence images of tissue sections showing microvessel in the ischemic limbs stained for platelet endothelial cell adhesion molecule 1 (CD31) at 14 days after surgery. B, Quantification of microvessel density showed significantly increased microvessel density in ischemic limbs treated with CEP03 alone (n=8) or CEP03 in conjunction with bFGF (basic fibroblast growth factor; bFGF+CEP03, n=7) compared with the control (n=6). Scale bar=100 μ m. * $P < 0.05$. CEP indicates ω -(2-carboxyethyl)pyrrole.

susceptibility to aggregation and degradation, and minimal benefit in clinical trials.^{4,26} In this study, we sought to pursue an alternative to angiogenic growth factors, namely, small molecules. By developing CEP-related chemical entities, we synthesized a novel small molecule that demonstrates significant proangiogenic activity. In comparison to CEP protein or peptide adducts, CEP03 is much smaller in size and amenable to further chemical modifications to optimize various drug properties, which is obviously advantageous for clinical development.

A few existing small molecules have been reported to have angiogenic effects in various assays and animal models.^{10,11} Desferoxamine, one of the most established iron chelators, activates HIF-1 (hypoxia inducible factor 1) and induces high-level expression of VEGF and erythropoietin mRNA.^{27,28} Desferoxamine was also demonstrated to increase tubelike structure formation on Matrigel with human umbilical vein ECs.²⁹ In addition, desferoxamine enhanced endothelial sprouting and vessel formation both in vitro and in vivo.²⁹ However, because iron is also a cofactor for oxidative phosphorylation and arachidonic acid signaling, a limitation of desferoxamine's translational potential is its off-target effects.³⁰ Resveratrol, a small molecule that constitutes the medicinal component of red grapes, was shown to have anti-inflammatory, antiapoptosis, reactive oxygen species

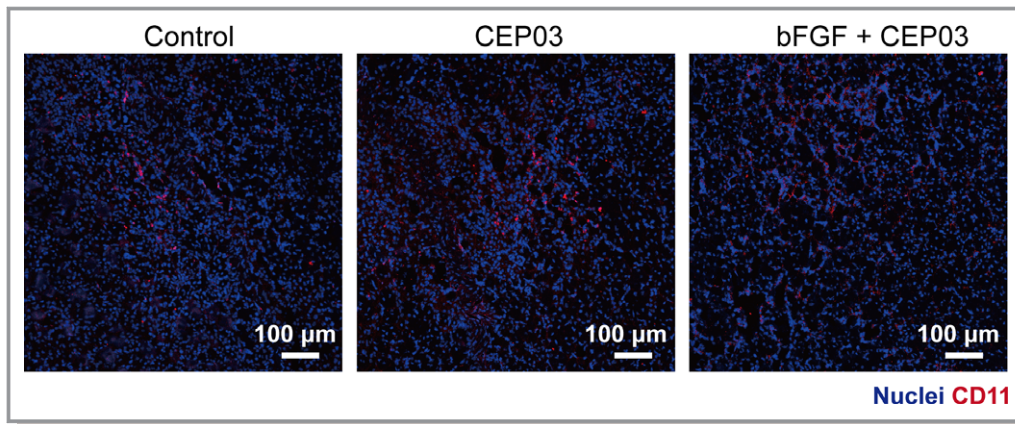


Figure 13. Immunofluorescence staining of CD11b (macrophage 1 antigen) for comparative analysis of inflammation. Representative images of CD11b in transverse sections of the ischemic limb show no significant difference between groups. bFGF indicates basic fibroblast growth factor; CEP, ω -(2-carboxyethyl)pyrrole.

scavenging, and angiogenic properties.³¹ Specifically, resveratrol enhanced EPC proliferation, migration, and adhesion and enhanced tube-like formation by increasing VEGF expression.³¹ However, resveratrol has opposing effects of being angiogenic at low concentration (2 ng/mL) but inhibiting cell survival and angiogenic pathways at high concentration (1.2 mg/mL).¹¹ Consequently, it may cause negative effects unless sustained at low dosage. Another small molecule, curcumin, was able to significantly upregulate CD31,³² E-selectin,³³ and both VEGF and VEGFR-2 when delivered serum-free.³⁴ Kiran et al showed that curcumin not only promoted capillary network formation in ECs but also increased vessel sprouting in aortic ring assays and enhanced vascular density in chick chorioallantoic membrane assay.³⁴ However, serum-containing curcumin application resulted in opposite, antiangiogenic effects.³⁴ In addition, either oral consumption or intraperitoneal injection of curcumin resulted in \approx 75% excretion, which suggested low absorption by the gastrointestinal tract.^{35,36} Given the limitations with existing small molecules, CEP03 may be an attractive alternative for translational application. Furthermore, in contrast to most reported angiogenic chemicals that are effective at the high concentrations (tens to thousands micromolar), our results indicated that CEP03 can be effective at the micromolar level.

Although the mechanism of action by which CEP03 enhances angiogenesis is largely unknown, a previous study showed that CEP01 induces angiogenic response by activation of the TLR signaling pathway.^{8,9} During wound healing, endogenously generated CEP-based factors directly interact with TLR2 in a MyD88 (myeloid differentiation primary response 88)-dependent manner, resulting in Rac1 (Rac family small GTPase 1) activation, and then facilitates integrin function. Intriguingly, our data demonstrated a TLR2-independent pathway. Instead, our results suggest that CEP03 acts in

an eNOS-dependent manner. Because eNOS and nitric oxide signaling are critical to the angiogenic function of ECs,^{37,38} it is reasonable that eNOS may be an important mediator of the effects of CEP03. Nevertheless, a more complete understanding of the basic signaling pathway and concentration-dependent effects is necessary toward clinical translation of this small molecule drug. Furthermore, additional studies of molecular mechanisms and structure-activity relationships will be helpful for better understanding CEP-based small molecules, particularly for drug development at a future stage.

In conclusion, we demonstrated that CEP03 enhances EC/EPC proliferation, tubelike formation, and wound-healing efficiency. In addition, delivery of CEP03 significantly improved blood flow perfusion recovery in the ischemic hind limbs of mice. The findings from this study have important implications for the delivery of small molecules for augmenting angiogenesis in ischemic tissues.

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Disclosures

None.

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