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20-HETE contributes to ischemia-induced angiogenesis

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ABSTRACT

Angiogenesis is an important adaptation for recovery from peripheral ischemia. Here, we determined whether 20-hydroxyeicosatetraenoic acid (20-HETE) contributes to ischemia-induced angiogenesis and assessed its underlying molecular and cellular mechanisms using a mouse hindlimb-ischemia angiogenesis model. Hindlimb blood flow was measured by Laser Doppler Perfusion Imaging and microvessel density was determined by CD31 and tomato lectin staining. We found that systemic and local administration of a 20-HETE synthesis inhibitor, DDMS, or a 20-HETE antagonist, 6,15-20-HEDGE significantly reduced blood flow recovery and microvessel formation in response to ischemia. 20-HETE production, measured by LC/MS/MS, was markedly increased in ischemic muscles (91 ± 11 vs. 8 ± 2 pg/mg in controls), which was associated with prominent upregulation of the 20-HETE synthase, CYP4A12. Immunofluorescence co-localized increased CYP4A12 expression in response to ischemia to CD31-positive EC in the ischemic hindlimb microvessels. We further showed that ischemia increased HIF-1α, VEGF, and VEGFR2 expression in gracilis muscles and that these increases were negated by DDMS and 6,15-20-HEDGE. Lastly, we showed that ERK1/2 of MAPK is a component of 20-HETE regulated ischemic angiogenesis. Taken together, these data indicate that 20-HETE is a critical contributor of ischemia-induced angiogenesis in vivo.

1. Introduction

Angiogenesis within the context of ischemia-induced compensatory neovascularization minimizes tissue damage caused by atherosclerotic diseases, including peripheral arteriopathy [1,2]. However, ischemic angiogenesis, like arteriogenesis, in patients with peripheral artery disease is often insufficient to maintain adequate tissue perfusion resulting in critical limb ischemia and amputation [3]. Thus, discovering novel factors which increase angiogenesis in response to ischemia in animal models of hindlimb ischemia is of potential clinical relevance.

20-Hydroxyeicosatetraenoic acid (20-HETE) is a vasoactive arachidonic acid (AA) metabolite produced by cytochrome P450 ω-hydroxylases (most notably of the CYP4A and CYP4F gene subfamilies in humans [4–7]). It is primarily found in the microcirculation [8,9]. Accumulating evidence shows that 20-HETE plays an important role in angiogenic processes [10–16]. 20-HETE has been shown to activate vascular endothelial cells (EC) and vascular smooth muscle cells (VSMC) by inducing their proliferation, migration, survival and tube formation as well as secretion of pro-angiogenic growth factors, such as vascular endothelial growth factor (VEGF) by EC and its upstream transcriptional factor hypoxia inducible factor 1α (HIF-1α) [11,12,17,18]. The HIF-1α/VEGF signaling pathway is essential for angiogenesis [19,20]. We recently reported that 20-HETE regulates angiogenic function of endothelial progenitor cells (EPC) in vitro and EPC-mediated angiogenesis in vivo [13,21]. Amaral and colleagues first reported that CYP4A, and by extension 20-HETE, play a critical role in angiogenesis induced by chronic electrical stimulation of skeletal muscle [15]. Jiang et al. have shown that CYP4A1 overexpression in smooth muscle promotes endothelial sprouting in renal arterial microvessels [22]. Furthermore, 20-HETE has been shown to induce angiogenic responses in rat cornea and HET0016 (N-hydroxy-N′-(4-butyl-2-methylphenol) formamidine), a selective 20-HETE synthase inhibitor, markedly reduced epithelial growth factor (EGF)-, VEGF-, and fibroblast growth factor (FGF)-induced angiogenic responses in the cornea [16]. These studies indicate that 20-HETE stimulates a variety of pro-angiogenic responses in cultured cells.

Abbreviations: AA, arachidonic acid; 20-HETE, 20-hydroxyeicosatetraenoic acid; EC, endothelial cells; HMVEC, human microvascular endothelial cells; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; HIF-1α, hypoxia-inducible factor-1-alpha; HET0016, N-hydroxy-N′-(4-n-butyl-2-methylphenyl) formamidine; DDMS, dibromo-dodecenyl-methylsulfimide; 6,15-20-HEDGE, N-(20-hydroxyeicosa-6(Z),15(Z)-dienoyl) glycine.

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However, it is unknown whether 20-HETE plays a role in ischemia-induced angiogenesis in vivo. Furthermore, the cellular and molecular mechanism(s) by which it may do so are completely unexplored. The present study was designed to investigate whether 20-HETE plays a central role in angiogenesis in the ischemic hindlimb and by which mechanism it does so.

2. Material and methods

2.1. Cell culture

Human microvascular endothelial cells (HMVEC) were purchased from Promo Cells (Heidelberg, Germany) and cultured according to the manufacturer's recommendations and maintained at 37 °C in a humidified incubator containing 5% CO_2 as previously described [11]. Cultures from two separate batches with passage numbers 3–4 were used. Prior to the experiments, cells were plated at 60% density in EC growth media (Promo Cells) and allowed to grow overnight before incubating under either normoxia (20% O_2) or hypoxia (3% O_2; GCA Precision Hypoxic Incubator) for 16 h.

2.2. Mouse hindlimb ischemia angiogenesis model

The mouse hindlimb ischemia model is a well-established in vivo angiogenesis model [23,24], in which angiogenesis occurs in the gracilis muscle, while arteriogenesis occurs proximal to the ligation, in the adductor muscle [25–30]. Twelve-week-old Balb/c mice were purchased from the Jackson Laboratory (Bar Harbor, MA). All animal experiments were performed according to the NIH guidelines using animal care and experimental protocols that were approved by the Institutional Animal Care and Use Committee at New York Medical College. Briefly, mice were anesthetized with ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg i.p.) as described previously [31] prior to unilateral (right) femoral artery ligation. The femoral artery was exposed through a 1 cm incision in the right inguinal region. Then, the artery was separated from the vein and nerve and ligated with a 4-0 suture proximal to the common femoral artery and distal to the bifurcation of the profunda and excised in between. The incision was closed after the wound was irrigated with sterile saline and topical antibiotic was applied after wound closure. The arteries on the left side were not ligated and served as control. At the end of each experimental time point, mice were sacrificed by CO_2 inhalation prior to tissue harvest for further analysis. An overall experimental protocol and design based on the mouse ischemia hindlimb angiogenesis assay used in this study is depicted in Supplemental Fig. S1.

2.3. Pharmacological 20-HETE interventions

20-HETE synthesis inhibitor, dibromo-dodecenyl-methylsulfimide (DDMS) and 20-HETE antagonist, N-(20-hydroxyeicosa-6(Z), 15(Z)-dienoyl) glycine (6,15-20-HEDGE) were synthesized by Dr. Falck and used to interfere with the synthesis or action of 20-HETE in the mouse ischemic hindlimb angiogenesis model. The concentrations of DDMS and 6,15-20-HEDGE we chose were based on several previous in vivo studies from our groups and the others. For systemic administration, mice were treated with vehicle (DMSO), DDMS (10 mg/kg/day; i.p.), or 6,15-20-HEDGE (10 mg/kg/day; i.p.) for 2 days prior to the unilateral femoral ligation. Treatment continued daily for the next 21 days. For local administration of DDMS and 6,15-20-HEDGE to hindlimb gracilis muscle, an Alzet mini-osmotic pump (model 2002 or 2004; Durect Corporation, Cupertino, CA) was filled with the drugs (5 mg/kg/day) and pre-conditioned in sterile 0.9% saline at 37 °C overnight and then was attached with polyethylene catheter tubing (an inside diameter (I.D.) 0.8 mm). Mice were anesthetized the next day and a small incision was made in the skin between the scapulae. Using a hemostat, a small pocket was formed by spreading the subcutaneous connective tissues apart. The pump was inserted into the pocket and the open end of the catheter was directed and anchored onto the left gracilis muscles by suturing 2 days prior to femoral artery ligation and drugs were continuously delivered for the entire duration of the experiment. For the 20-HETE rescuing experiment, animals were treated with DDMS (10 mg/kg/day; i.p.) to inhibit systemic 20-HETE synthesis for 2 days prior to the unilateral femoral ligation. DMSO was used as vehicle control. Synthetic 20-HETE (5 mg/kg/day) (Cayman Chemical, Ann Arbor, MI) was delivered to the ischemic gracilis muscle in the presence and absence of DDMS via osmotic pump on the day of ligation.

2.4. Assessment of blood flow

All mice were anesthetized with 1% isoflurane and placed on a heat pad at 37 °C. Excess hindlimb hair was removed by depilatory cream before analysis. Blood flow in both limbs was measured using a Laser Doppler Perfusion Imaging (LDPI) scanner, PeriScan PIM3 (PeriMed, Jarfalla, Sweden) [31] before surgery (day 0) and then 1 day after the surgery to confirm the surgical outcome of limb ischemia. Then, LDPI was continued in DMSO-, DDMS-, 6,15-20-HEDGE-, and/or 20-HETE treated mice on days 1, 7, 14, and 21 post-ligation to quantify hindlimb blood flow in both legs. A region of interest (ROI) was created around each limb relative to the anatomical marker (mid-inguinal point-between iliac crest and symphysis pubis) and the ratio of blood perfusion in the ischemic limb vs. the non-ischemic limb was calculated.

2.5. Measurement of blood pressure

Systolic blood pressure measurements were taken via the CODA tail-cuff system (Kent Scientific), which utilizes volume pressure recording sensor technology. Separate groups of vehicle-, DDMS- and 6,15-20-HEDGE treated mice were acclimated to the machine for 1 week prior to day 0 and blood pressure was monitored throughout the length of the experiment, at days 1, 7, 12, 14, 21, 26, 28 and 32 post ligation as previously described [32]. Twenty individual readings were taken and recorded. Values within $\pm$10% of their mean blood pressure measurements were obtained.

2.6. Histology and immunofluorescent (IF) microscopy

For microvessel density (MVD) analysis, hindlimb gracilis muscles from control and experimental groups were surgically excised at the end of experiments (day 21), frozen sectioned (6 μm). Microvessels were identified and labeled by double IF staining against two different endothelial markers, CD31 and tomato lectin. Briefly, sections were washed with 0.1% Tween-20/PBS 3× and non-specific reactions were blocked with 5% BSA/0.3 M glycine/0.1% Tween-20/PBS (blocking buffer) at room temperature (RT) for 2 h. After blocking, the sections were incubated with the primary antibody rat anti-mouse CD31 (1:100) (eBioscience, San Diego, CA) at 4 °C overnight followed by anti-rat Cy3-conjugated secondary antibodies (1:500) (Jackson Immunoresearch, West Grove, PA) for 3 h at RT. The samples were thoroughly washed and then treated with 10 mg/ml tomato lectin-FITC (Vector Laboratory, Burlingame, CA) at RT for 30 min, followed by 0.1 mg/ml DAPI (Sigma-Aldrich, St. Louis, MO, USA) counterstain for 10 min. Images were acquired using the Zeiss AXIO Imager M1 fluorescence microscope. Six–eight fields were chosen randomly from various sections to ensure objectivity. CD31 and tomato lectin double positive microvessels were counted. The number of muscle fibers was also counted within the same field on bright field images. MVD data is shown as the microvessel to muscle fiber ratio in each field.

For analysis of arterioles, rat anti-α-smooth muscle actin (1:250; VSMC markers; eBioscience) was used to stain for VSMCs in combination with anti-rat FITC-conjugated secondary antibodies (1:1000). IF images from 6–8 randomly chosen fields were captured using the Zeiss AXIO Imager M1 fluorescence microscope. α-SMA positive

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