Neutrophil-derived Myeloperoxidase and Hypochlorous Acid Critically Contribute to 20-HETE Increases that Drive Post-Ischemic Angiogenesis

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Abstract

Compensatory angiogenesis is an important adaptation for recovery from critical ischemia. We recently identified 20-hydroxyeicosatetraenoic acid (20-HETE) as a novel contributor of ischemia-induced angiogenesis. However, the precise mechanisms by which ischemia promotes 20-HETE increases that drive angiogenesis are unknown. This study aims to address the hypothesis that inflammatory neutrophil–derived myeloperoxidase (MPO) and hypochlorous acid (HOCl) critically contribute to 20-HETE increases leading to ischemic angiogenesis. Using LCMS/MS/MS, Laser Doppler Perfusion Imaging (LDPI), and Microvascular Density (MVD) analysis, we found that neutrophil depletion and MPO knockout mitigate angiogenesis and 20-HETE production in the gracilis muscles of mice subjected to hindlimb ischemia. Furthermore, we found MPO and HOCl to be elevated in these tissues post-ischemia as assessed by immunofluorescence microscopy and in vivo live imaging of HOCl. Next, we demonstrated that the additions of either HOCl or an enzymatic system for generating HOCl to endothelial cells increase the expression of CYP4A11 and its product, 20-HETE. Finally, pharmacological interference of hypoxia inducible factor (HIF) signaling results in ablation of HOCl-induced CYP4A11 transcript and significant reductions in CYP4A11 protein. Collectively, we conclude that neutrophil-derived MPO and its product HOCl activate HIF-1α and CYP4A11 leading to increased 20-HETE production that drives post-ischemic compensatory angiogenesis.
**Significance Statement.** Traditionally, neutrophil derived MPO and HOCl are exclusively associated in the innate immunity as potent bactericidal/virucidal factors. The present study establishes a novel paradigm by proposing a unique function for MPO/HOCl as signaling agents that drive critical physiological angiogenesis by activating the CYP4A11-20-HETE signaling axis via a HIF-1α-dependent mechanism. The findings from this study potentially identify novel therapeutic targets for the treatment of ischemia and other diseases associated with abnormal angiogenesis.
Introduction

Ischemia elicits compensatory angiogenesis to restore vitality to injured tissues, but often fails to adequately perfuse these tissues leading to chronic and life-threatening disease (Idris et al., 2004; Vouillarmet et al., 2016). Consequently, much effort has and continues to be expended to discover novel factors that contribute to the formation of collateral vessels in response to ischemia. Identifying these factors is critical to developing therapies for the treatment of cardiovascular diseases.

20-HETE is the ω–carbon hydroxylation product of arachidonic acid (AA) catalyzed by the CYP monooxygenases of the CYP4A and 4F subfamilies. 20-HETE is found in many tissues and organs including the kidney, liver, lung and brain (Omata et al., 1992; Harder et al., 1994; Birks et al., 1997; Ito et al., 2006). It has mitogenic properties and is recognized for its role in hypertension and renal function (Miyata and Roman, 2005; Spector and Norris, 2007; Hoopes et al., 2015). Recently, CYP4A11/20-HETE is emerging as a major contributor of angiogenesis (Chen et al., 2012, 2014, 2016, 2019). 20-HETE participates in both autocrine and paracrine signaling to coordinate the major events in angiogenesis, including the proliferation and migration of the endothelial cells that line nascent blood vessels (Jiang et al., 2004; Guo et al., 2007, 2009, 2011; Dhanasekaran et al., 2009). 20-HETE also promotes the survival of these cells by inhibiting apoptosis (Wang et al., 2008; Dhanasekaran et al., 2009). 20-HETE induces these aforementioned actions by the activation of the MAPK and PI3K pathways (Chen et al., 2016) and participates in a positive feedback mechanistic loop with vascular endothelial growth factor (VEGF) (Amaral et al., 2003; Jiang et al., 2004; Chen et al., 2005, 2014; Guo et al., 2007, 2009). Our lab recently demonstrated that
endogenous 20-HETE critically contributes to angiogenesis following ischemic injury in a mouse hindlimb angiogenesis model (Chen et al., 2016, 2019), based on two seminal findings: 1) pharmacological interference of the synthesis or the actions of 20-HETE attenuate hindlimb angiogenesis post-ischemia; and 2) the production of 20-HETE is markedly elevated in ischemic hindlimb tissues. However, the precise cellular and molecular mechanisms which cause 20-HETE to increase which ultimately drive post-ischemic angiogenesis are completely unknown.

The vascular endothelium responds rapidly to changes in oxygen tension and nutrients (Shweiki et al., 1992). In response to ischemic insult, endothelial cells produce and release cytokines (Lykov et al., 2019) which recruit inflammatory cells to sites of injury. In humans, neutrophils are the most abundant circulating leukocytes with a high rate of production and turnover (Lahoz-Beneytez et al., 2016) and can rapidly respond to the injured endothelium. Neutrophils have long been associated with post-ischemic angiogenesis (Noonan et al., 2008; Gong and Koh, 2010). These cells are well suited to participating in this process because they are highly mobile and are able to access sites of injury (Phillipson et al., 2006). At these sites, neutrophils act to remodel the extracellular matrix (ECM) to allow for the expansion of nascent blood vessels (Hawinkels et al., 2008). Neutrophils remodel the ECM by releasing proteases and MPO from their primary granules, as well as by generating superoxide and hydrogen peroxide ($H_2O_2$) (Lacy, 2006; Sheshachalam et al., 2014). MPO reduces a portion of this $H_2O_2$ to H$_2$O while oxidizing chloride to HOCl: the strongest oxidant deliberately formed by the body (Winterbourn and Kettle, 2013). MPO is widely implicated in a variety of vascular pathologies including atherosclerosis (Nicholls and Hazen, 2005; Castellani et al., 2006;
Schindhelm et al., 2009), coronary artery disease (Teng et al., 2017) and is used as a biomarker for predicting acute myocardial infarctions (Schindhelm et al., 2009; Tang et al., 2009). MPO is also increasingly recognized as a key regulator of neovascularization (DeNichilo et al., 2015; Panagopoulos et al., 2015; Khalil et al., 2018).

Despite the central role of 20-HETE in ischemia-induced angiogenesis, the mechanisms regulating its synthesis remain largely unknown. What is known is that ischemia causes cells to become hypoxic which upregulates the expression of HIF and CYP4A11 and increases 20-HETE production in endothelial cells (Chen et al., 2016). In many cases, hypoxia-induced HIF activation occurs rapidly following vessel blockade (Wang et al., 1995; Chen et al., 2016). Although, the HIF pathway can also be induced by ROS (Chandel et al., 2000; Hagen, 2012). Powerful oxidants such as HOCl may also activate the HIF pathway independent of hypoxia, thereby inducing CYP4A11 and 20-HETE synthesis.

In the current study, we present novel findings strongly supporting our central hypothesis: 20-HETE critically regulates ischemia-induced angiogenesis via neutrophil-derived MPO and HOCl-dependent activation of HIF-1α and CYP4A11.
Materials and Methods

Mouse Hindlimb Ischemia Angiogenesis Model. Twelve-week-old male immuno-competent Balb/C, C57BL/6J and B6.129X1-Mpo<tm1Lus>/J (MPO<sup>−/−</sup>) were purchased from the Jackson Laboratory (Bar Harbor, MA). The animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee at New York Medical College. The mouse hindlimb ischemia angiogenesis model was established to study the formation of new blood vessels as a result of ischemia induced by unilateral (right) ligation of the femoral artery (Carmeliet et al., 1998; Couffinhal, 2009). The mice are anesthetized with a ketamine (80 mg/kg, i.p.) and xylazine (10 mg/kg i.p.) cocktail and the hindlimb regions are placed under a microscope (standard 10× eyepiece magnification). A 1 cm incision is made in the right inguinal region and the femoral artery is identified and isolated from nerve and vein. The artery is ligated using a 4-0 size suture (Ethicon, Somerville, NJ) proximal to the common femoral artery and distal to the bifurcation of the profunda and excised in between. The wound is irrigated prior to closure and a topical antibiotic is applied after the wound is sutured. The artery on the contralateral (right) limb is not ligated and serves as the non-ischemic control. The animals are closely monitored throughout the 21-day experiments for potential infections and signs of discomfort and pain. At day 21, both ischemic and non-ischemic gracilis muscles from the hindlimbs were dissected for immunohistochemistry. The detailed methodology is well published by our group (Chen et al., 2016, 2019).

Laser Doppler Perfusion Imaging. Ischemia-induced angiogenesis was measured based on the total blood perfusion in the local microcirculation through the scanning...
motion of a laser beam. The blood perfusion of the ligated and non-ligated control limb of each mouse was measured at various times post-femoral artery ligation. Readings obtained from these time points were normalized to their day 0 measurement to account for individual differences in basal blood flow. The blood perfusion measurement for each mouse is represented as the ratio of the blood flow of the ligated (ischemic) limb over the non-ligated (non-ischemic) control limb. Based on these measurements, blood perfusion recovery is assessed at 21 days post-ischemia, when the most dramatic differences in angiogenesis can be observed.

**Immunofluorescent (IF) Microscopy.** To quantify micro-vessel density, the ischemic and non-ischemic contralateral gracilis muscles of each mouse were harvested on days 21 post-ligation. The excised muscles were snap frozen in liquid nitrogen and frozen sectioned (7 μM). The sections were incubated with rat anti-mouse CD31 (endothelial markers) (1:100) (Abcam, Cambridge, MA) overnight, followed by anti-rat Cy3-conjugated secondary antibodies (1:500) (Jackson Immunoresearch, West Grove, PA) for 3 hr at RT. The sections were then stained with Tomato Lectin, and DAPI was used as counter stain for nuclei. The number of CD31+ and Tomato Lectin+ micro-vessels were quantitated and normalized to the number of muscle fibers. The micro-vessel density of a sample is represented as the average of counts obtained from six random fields selected by a blinded observer. In addition, separate sets of frozen gracilis muscle sections were obtained and prepared 16 hr post-ligation and incubated with polyclonal goat anti-human/mouse MPO (1:250) (R&D systems, Minneapolis, MN) overnight, followed by incubation in anti-goat FITC-conjugated secondary antibodies (1:1000)
(Jackson Immunoresearch). Differential deposition of MPO in ischemic vs. non-ischemic hindlimb gracilis muscle was assessed based on the FITC green fluorescence.

**LC/MS/MS Analysis.** For *in vivo* experiments, the gracilis muscle of the ischemic and non-ischemic limb were excised on day 3 post-ligation, at the peak of 20-HETE production in these tissues (Chen *et al.*, 2016). The muscles were homogenized in oxygenated Kreb's buffer with a glass homogenizer on ice. Homogenates were then incubated with 1 mM NADPH (Calbiochem, San Diego, CA) for 30 min at 37°C in Kreb's buffer. For *in vitro* experiments, cultured human dermal microvascular endothelial cells (HDMEC) were treated with MPO (1.25 μg/ml) (Millipore Sigma, St Louis, MO) and HOCl (15 μM) for various times. Both cell media and cell lysates were collected at 16 hr post-treatment at the peak of CYP4A11 protein expression. Protein concentrations from cell lysates and muscle samples were determined using Pierce BCA protein assay kit (ThermoScientific, Rockford, IL). Tissue and cell samples were acidified to pH 4.0 using 10% acetic acid. The lipids were extracted 2x with ethyl acetate in the presence of d6-20-HETE as an internal standard (0.5 ng) (Cayman Chemical, Ann Arbor, MI). The organic phase was collected and dried under nitrogen. 20-HETE production was quantified with a Shimadzu UFMS Triple Quadrupole Mass Spectrometer LCMS-8050 combined with a Nexera UHPLC using negative ionization MRM mode. This ultra-sensitive method achieves 1 pg 20-HETE as the limit of quantitation. The details of analytical conditions are previously published by our group (Chen *et al.*, 2016). Final 20-HETE quantitation in tissues was normalized by 30-min reaction time over the total amount of mg protein/sample.
Neutrophil and Macrophage Depletion. Neutrophils and macrophages were systemically depleted using previously published methods (Abbitt et al., 2008; Bellner et al., 2015). In brief, mice were either treated with Ly6G/C antibody (i.p.; 0.5 mg) (BioXCell, Lebanon, NH) or Clodronate Liposomes (i.p.; 5 mg/ml, 200 μl) (Liposoma, Amsterdam, NL) 2 days prior to unilateral femoral artery ligation. Mice were injected every 3 days following ligation over the course of 21 days to maintain a robust depletion of neutrophils and macrophages. Saline or PBS-containing liposomes were administered in respective control mice.

Flow Cytometry Analysis. Neutrophil depletion was confirmed by isolating peripheral circulating blood from both untreated control and mice treated with LY6G/C antibody at days 0, 1, 3, 7, and 14 days post-ischemic ligation. Trucount flow cytometry experiments were performed on 25 μl of total blood directly stained with anti-CD11b (Abcam, Cambridge, MA) and anti-Gr-1 (Bio-Rad, Hercules, CA) antibodies washed into 2 ml of PBS then mixed with 25 μl of countBright absolute counting beads (Invitrogen) and resuspended into 2 ml of PBS before acquisition on a LSR II flow cytometer (BD Biosciences) by NYMC flow cytometry core. Live cells were gated based on their forward and side angle light scatter and analyzed using CellQuest Pro software (BD Biosciences). Neutrophil quantitation was expressed as CD11b⁺Gr-1⁺ (x10⁶) cells per ml of blood.

In Vivo and In Vitro Imaging of HOCl. For in vivo live imaging of HOCl generation, femoral artery ligation was performed to induce ischemia on the right hindlimb of C57BL/6J mice. A mock surgery was carried out on the contralateral left hindlimb, which serves as the non-ischemic control. In parallel, two additional groups of animals were
injected with either a purified HOCl solution (10 μL; 50 μM) or lipopolysaccharide (LPS) (10μL; 1 mg/mL) (Millipore Sigma) for 30 min in the gracilis muscle of their left hindlimbs. In all experiments, sterile saline (10μL) was injected in the contralateral right limb as a control. Detection of HOCl generation in hindlimb tissues was assessed and quantitated by subsequently injecting a HOCl-specific fluorescent probe: FDOCl-1 (Wei et al., 2018) (10 μL; 1 mM) in both hindlimbs. Fluorescence generated by the FDOCl-1 deformylation product was then detected 5 min after FDOCl-1 injections using the Xenogen In-vivo Imaging System (IVIS) Spectrum from Caliper Life Sciences (λ_em = 720nm, λ_ex = 640nm). MPO^-/- mice injected with FDOCl-1 in both hindlimbs were used as negative controls. All images were captured using the Living Image Software (IVIS Imaging Systems, Shelton, CT) under settings recommended by the manufacturer. FDOCl-1 fluorescence was analyzed and quantified using the ImageJ software (NIH).

For in vitro HOCl imaging, HDMEC were pre-incubated in 10 μM FDOCl-1 (Wei et al., 2018) for 1 hr and with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA) nuclear stain for 30 min. Cells were washed and incubated in HOCl (20 μM) for a total period of 10 min during which the cells were imaged live. Fluorescence due to FDOCl-1 deformylation was visualized in the red channel (λ_em = 700nm, λ_ex = 633nm) and nuclear fluorescence was visualized using the DAPI channel (λ_em = 460nm, λ_ex = 360nm). Images were taken using the Zeiss inverted Axiovert 200 motorised microscope using a 10x objective lens and analyzed using the NIS-Elements Viewer software (Nikon Instruments, Inc, Melville, NY).

Cell Culture. HDMEC were cultured in Endothelial Cell Basal Medium 2 supplemented with Endothelial Cell GM MV2 in conditions recommended by the manufacturer.
(PromoCell, Heidelberg, Germany). HDMEC were maintained at 37°C in a humidified incubator containing 5% CO₂. Passages numbers 5-6 were used for all the experiments shown.

**In vitro MPO and HOCl Studies.** For MPO incubation studies in HDMEC, MPO (1.25 μg/ml) (Millipore Sigma) was co-incubated with glucose oxidase (GOx; 50 μg/ml) (Millipore Sigma) and glucose (250 μM) to generate 10 μM HOCl in cell culture. These conditions were optimized to maintain HDMEC viability in the presence of this system. Hydrogen peroxide concentrations were determined using the FOX method (Banerjee *et al.*, 2003; Meisner and Gębicki, 2009). We developed an assay for the determination of HOCl concentrations which was carried out in 1mL reaction volumes containing 10 μM of FDOCl-1 (Wei *et al.*, 2018). This assay reliably measures HOCl concentrations in the 5-30 μM range. HDMEC were incubated in MPO alone, or MPO with GOx and glucose for 15 min to generate 10 μM HOCl.

For HOCl incubation experiments in HDMEC, sodium hypochlorite stock (Millipore Sigma) concentration was determined by the NTB method (Jeitner *et al.*, 2013) and optical density at 292 nm (Hussain *et al.*, 1970). HOCl spectra and colorimetric analyses were performed in a 1:1000 dilution of HOCl stocks in quartz cuvettes. HDMEC were incubated in HOCl (15 μM) for 5 min in a volume of 5 ml PBS. All remaining HOCl present in the incubation media was quenched using spent media and cells were subsequently washed and incubated in serum-free basal media.

**HIF-Luciferase Reporter Transfection and Assay.** HDMEC were transfected using a Cignal HIF Pathway Luciferase Reporter Assay kit (Qiagen) at a multiplicity of infection (MOI) of 10 viral particles/cell. Transfections were carried out over 24 hr in the presence
of SureENTRY Transduction Reagent (10 μg/mL) (Qiagen) in low serum conditions (0.5%) as recommended by the manufacturer. Transfection conditions were optimized in HDMEC transfected with the appropriate Cignal Positive Control Assay (Qiagen) which promotes constitutive expression of GFP. Successful transfections were confirmed by GFP positive fluorescence using a Zeiss inverted Axiovert 200 motorised microscope. Successful transfections with HIF-luciferase reporter were confirmed in HDMEC incubated in hypoxic conditions (5% O₂, 4 hr) or cobalt (II) chloride (CoCl₂) (100μM, 16 hr). HDMEC were cryopreserved following successful transfections.

Next, HDMEC successfully transfected with the HIF-luciferase reporter were incubated in 15 μM HOCl for 5 min and harvested after various times (15, 30, 60, 120, and 240 min) using luciferase assay lysis buffer (Thermo Fisher – Cat No. 16189) in the presence of a protease inhibitor (Sigma – Cat No. 539131). Cell lysates (10 μl) were incubated in luciferase assay buffer (90 μl) (Pierce Firefly Luciferase Glow Assay Kit – Thermo Fisher Scientific) for 10 min at RT. The production of oxyluciferin product was detected by the luminometry capability of the SpectraMax ID5 microplate reader (Molecular Devices, San Jose, CA). Relative luciferase expression was determined based on luminescence normalized to lysate protein content.

**Real-time PCR.** HDMEC were harvested with RLT Lysis Buffer (Qiagen, Hilden, Germany) containing 1% BME and RNA was isolated using the RNeasy extraction kit (Qiagen). RNA was quantified using Take 3 Micro-Volume Plate and 500 ng of RNA was loaded for cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Newly synthesized cDNA was loaded onto a 96-well PCR plate and incubated with Taqman Master Mix (Thermo Fisher Scientific) and the
following FAM-MGB probes (assay IDs shown in parentheses): CYP4A11 (Hs04194779_g1; Thermo Fisher Scientific) and GAPDH (Hs02786624_g1; Thermo Fisher Scientific). RT-PCR was performed using the QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 50°C for 2 min. The threshold cycle (Ct) data was determined using default threshold settings. The Ct was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in the gene expression (Livak and Schmittgen, 2001).

**HIF Inhibitor Studies.** HDMEC were pre-incubated with HIF inhibitors V (HIFI-V: 0.5 μM; Calbiochem, Sigma) and SCBT (sc205346: 0.5 μM; Santa Cruz Biotechnology, Santa Cruz, CA) for 16 hr. Cells were subsequently treated with HOCl (15 μM) for 5 min and harvested at times of maximal CYP4A11 mRNA (1 hr) and protein (16 hr) expression for comparisons. Effective concentrations of HIF inhibitors are based on previous findings (Naik *et al.*, 2012; Yamazaki *et al.*, 2017) and cell viability in coincubations with HOCl.

**Western Blot Analysis.** HDMEC were incubated in: 1) MPO (1.25 μg/mL) or 2) MPO (1.25 μg/mL) + GOx (50 μg/mL) + Glucose (250 μM) for 15 min to generate 15 μM HOCl or 3) 15 μM HOCl for 5 min. Cells were then incubated in basal media and harvested with RIPA buffer containing protease inhibitor (Sigma – Cat No. 539131) after various time points (30 min, 1hr, 2hr, 4hr, and 16 hr). Lysates were quantified and equal amount of protein lysates (25 μg) were loaded and separated on a 12% Tris-glycine gel. Proteins were transferred to a PVDF membrane using a methanol-glycine-tris base
buffer overnight. Next, membranes were incubated in anti-CYP4A11 (Sigma-SAB2702082) (1:2500), anti-HIF1α (Abcam-ab16066) (1:500) at 4 °C overnight followed by incubation with anti-rabbit IRDye 800 CW secondary antibody (Cat. No - 926-32211) or anti-mouse IRDye 680 RD secondary antibody (Cat. No - 926-68070) (1:10,000) (LI-COR, Lincoln, NE) at RT for 2 hr. Antibodies were validated and used as described in our previous publications (Guo et al., 2009; Chen et al., 2019). The blots were then scanned with LI-COR Odyssey Infrared Imaging System. β-actin (Santa Cruz Biotechnology SC47778) (1:10,000) was used as a loading control. All western blots were quantitated using NIH ImageJ software by comparing the density ratios of control band/β-actin to experimental band/β-actin. HIF-1α and CYP4A11 antibodies were individually validated in house (Supp. Fig. 1).

**Statistical Analysis.** Data analyses were performed using the GraphPad Prism 9 software. In all *in vitro* cell culture experiments, data is expressed as Means ± SD. In all *in vivo* animal experiments, data is expressed as Means ± SEM. Statistically significant differences were determined using one-way ANOVA analyses, one-way ANOVA analyses with repeated measures, and unpaired and paired t-tests. Tukey post-hoc test was used for multiple comparisons between groups and Dunnett post-hoc test was used for multiple comparisons between experimental and control groups. p<0.05 was considered statistically significant. All n values represent individual biological replicates. Data expressed with multiple n values represent biological replicates that were collected in separate experiments and conducted alongside their respective controls.
Results

Neutrophil-derived MPO contributes to post-ischemic 20-HETE increases leading to 20-HETE-regulated angiogenesis. Ischemia can quickly recruit inflammatory cells to the site of injury (Movat et al., 1987; Mayadas et al., 2014). To test the hypothesis that inflammatory leukocytes contribute to 20-HETE-regulated angiogenesis, we depleted C57/BL mice of neutrophils or macrophages before and after femoral artery ligation by administrating either Ly6G/C antibodies or clodronate liposomes, respectively. Specific and persistent neutrophil depletion of greater than ~75% in these animals by days 3 post-ischemia was achieved and confirmed by flow cytometric analysis of Gr-1+ and CD11b+ neutrophils (Fig 1A). The administration of Ly6G/C antibody also resulted in a modest reduction of CD115+CD11b+ circulating monocytes (~20%) due to off-target depletion (Supp. Fig. 2). LC/MS/MS analysis showed a 4-5-fold increase of 20-HETE production in the excised ischemic hindlimb muscles of both the control and macrophage-depleted mice compared to the corresponding contralateral non-ischemic hindlimb muscles (Fig 1B). Importantly, 20-HETE levels fail to increase in ischemic hindlimb gracilis muscles of mice selectively depleted of neutrophils (Fig 1B). Depleting the mice of macrophages alone, does not affect the rise in gracilis muscle 20-HETE amounts caused by ligation of the femoral artery (Fig 1B). By contrast, transgenic mice with MPO globally deleted (MPO−/−) show abrogated 20-HETE increases in their hindlimb ischemic tissues (Fig 1B). Moreover, blood flow perfusion recovery of neutrophil-depleted as well as MPO−/− mice post-ischemia are significantly reduced at the end of the 21-day experiment compared to their corresponding controls (Fig 1C & D). These findings in blood perfusion recovery are
consistent with a similar reduction in the number of micro-vessels present in the hindlimb gracilis muscles of these animals at days 21 post-ligation (Fig 1E).

**MPO and HOCl are present and detectable in ischemic hindlimb gracilis muscles.**

Having established the primacy of neutrophils and MPO in the angiogenic response to ischemia, we sought to confirm the deposition of MPO in the injured tissues. Neutrophil infiltration and MPO deposition in response to injury such as ischemia are dynamic processes (Ley et al., 2007). MPO staining using immunofluorescence microscopy shows that MPO is present and deposited in the ischemic gracilis muscles within 16 hr of femoral artery occlusion (Fig 2A). No such deposits are evident in the contralateral non-ischemic control limbs. In the process of reducing H$_2$O$_2$ to H$_2$O, MPO can oxidize a variety of halides and the pseudohalide thiocyanate to their corresponding hypohalous acids (Harrison and Schultz, 1976; Senthilmohan and Kettle, 2006; Davies, 2011). Chloride predominates as the most available substrate for MPO-catalyzed oxidation in blood and muscle and leads to the formation of HOCl (Van Dalen et al., 1997; Chapman et al., 2009). Therefore, we investigated the production of HOCl in ischemic hindlimbs using the IVIS real-time *in vivo* imaging by employing a HOCl-specific fluorescent probe, FDOCl-1 (Wei et al., 2018). Administration of the FDOCl-1 probe (1 mM in 10 µl) into the ischemic and non-ischemic hindlimbs of mice 30 min post-ischemia results in a rapid and significant increase in fluorescence activity in the ischemic hindlimbs compared to the contralateral non-ischemic control hindlimbs (Fig 2B). The magnitude of the ischemia-induced FDOCl-1 reactivity was compared to the hindlimbs of mice injected with lipopolysaccharide (LPS; 10 µg in 10 µl), a potent inducer of neutrophil activation (Richer et al., 2018) and reagent HOCl (25 µM in 10 µl), which were used as positive controls.
To confirm that HOCl is produced endogenously by MPO in these tissues, we performed the same experiment in MPO−/− mice. FDOCl-1 fluorescence was not detected in either hindlimb (Supp. Fig. 3) of MPO−/− animals, suggesting that ischemia fails to induce HOCl production in the absence of MPO.

**HOCl and catalytically active MPO significantly promotes CYP4A11 expression leading to 20-HETE production in HDMEC.** Endothelial cells are amongst the first vascular cell population which interact with circulating neutrophils and thus are involved in the initial responses secondary to ischemia. To better understand how HOCl signals the production of 20-HETE, we first investigated the interaction of HOCl and cultured HDMEC preloaded with FDOCl-1. In these experiments we added HOCl at a concentration of 20 μM for 10 minutes, which is a physiologically relevant (2x10^6 neutrophils/mL produce 50 μM HOCl in 30 minutes) (Klebanoff, 1970; Dypbukt et al., 2005) and is a non-toxic amount (data not shown). FDOCl-1 fluorescence in these cells develops within minutes of HOCl exposure, consistent with a rapid entry of HOCl into the cells (Fig 3A). By 10 min, the cells exhibit maximal FDOCl-1 fluorescence. Based on the known reactivity of HOCl with cysteinyl and methionyl residues (Folkes et al., 1995; Pattison and Davies, 2001; Winterbourn et al., 2016) and the role of MPO in 20-HETE regulated angiogenesis (Fig. 1), we hypothesized that HOCl reacts with intracellular moieties to activate the main human 20-HETE synthase, CYP4A11. Consequently, we examined the levels of CYP4A11 transcript, CYP4A11 protein, and 20-HETE production in HDMEC incubated with HOCl (15 μM) and MPO (1.5 μg/ml). We uncovered a time-dependent increase of CYP4A11 transcript as early as 30 min post-HOCl exposure and the response peaks after 1 hr (Fig 3B). Similarly, CYP4A11 protein
also markedly increases as early as 1 hr and its expression peaks at 16 hr post-treatment (Fig 3C). Interestingly, we found that MPO on its own has no significant effects on CYP4A11 transcript (Fig 3D), protein (Fig 3E), as well as 20-HETE production (Fig 3F) in HDMEC. Since MPO is catalytically inactive in the absence of H$_2$O$_2$, HOCl is not produced under these conditions (Davies, 2011; Winterbourn et al., 2016). We tested the effects of combining MPO and an enzymatic source of hydrogen peroxide (glucose oxidase (50 µg/ml) acting on glucose (250 µM): GOx) on promoting CYP4A11 synthesis. As expected, we found that catalytically active MPO significantly elevates both CYP4A11 transcript (Fig 3D) and protein (Fig 3E). More importantly, these changes in CYP4A11 also correspond to changes in enzymatic activity, as evidenced by increases in the amounts of 20-HETE produced by reagent HOCl and HOCl produced by MPO and GOx (Fig 3F). We also determined the effects of basal H$_2$O$_2$ generated by glucose/glucose oxidase alone on CYP4A11. Though glucose oxidase/glucose elevates CYP4A11 transcript (1.46-fold ± 0.18) and protein (1.56-fold ± 0.10) (Supp. Fig. 4), these changes are significantly reduced compared to the changes induced by HOCl generated by MPO (transcript: 2.86-fold ± 0.26, protein: 2.24-fold ± 0.56). Lastly, the optimal concentrations of H$_2$O$_2$ and HOCl generation were established as shown in Supp. Fig. 5.

**HOCl promotes the expression of HIF-1α protein and HIF transcriptional signaling in HDMEC.** Powerful oxidants such as HOCl may act as initiating and potentiating factors in the induction of the HIF signaling pathway independent of hypoxia (Chandel et al., 2000; Hagen, 2012), in turn inducing CYP4A11 and 20-HETE synthesis. Thus, we examined the effects of HOCl on the expression of HIF-1α protein in HDMEC. Fig 4A
showed that HIF-1α protein is significantly elevated in HDMEC as early as 30- and 60-min following exposure to HOCl (15 μM). While HIF-1α amounts reflect the potential for HIF dimerization and activation, HIF-dependent transcriptional activity is a more reliable indicator of the ability of this system to activate the transcription of specific genes, such as those encoding the CYP4A11 ω-hydroxylases. The measurement of HIF transcriptional activity also integrates the activation of other HIF isoforms (HIF-2 and/or HIF-3) that may be affected and contributing to the overall activation of hypoxia responsive element (HRE)-target genes. Consequently, we transfected HDMEC with a luciferase reporter at multiple HRE promoter regions to assess the effects of HOCl on this transcription factor. Exposure of these transfected HDMEC to HOCl results in significant luminescence increases due to HIF-dependent transcription of luciferase, within 30 minutes of HOCl exposure and the luminescence peaks at 2 hr in these cells (Fig 4B). The observed temporal differences in HIF1α protein and HIF transcriptional upregulation are consistent as transcriptional activity is expected to occur following the accumulation of HIF1α.

**HOCl-induced CYP4A11 transcript and protein are HIF-dependent.** The actions of HOCl on HIF-dependent signaling were confirmed using two inhibitors of this signaling and measuring CYP4A11 mRNA and protein in HDMEC. The inhibitors are methyl 3-[[2-[4-(2-adamantyl) phenoxy]acetyl]amino]-4-hydroxybenzoate (SCBT), which reduces HIF-1α accumulation and gene transcriptional activity (Yamazaki et al., 2017), and (E)-3-(3-(4-Adamantan-1-ylphenoxy)acrylamido)benzoic acid morpholinoethyl ester (HIFI-V), which upregulates the Von Hippel-Lindau (pVHL) suppressor gene product and promotes HIF1α degradation (Naik et al., 2012). The mechanisms of their actions are
illustrated as in Fig 5A. Treatment of HDMEC with SCBT abolishes the increases in CYP4A11 mRNA and protein caused by exposure of these cells to HOCl (Fig 5 B-C). HIFI-V also abolishes the HOCl-induced increases of CYP411 mRNA (Fig 5 B-C). The effect of this inhibitor on the inductions of CYP4A11 protein by HOCl are less pronounced but still significant (Fig 5C).
Discussion

The CYP4A-20-HETE axis is a novel regulator of pathological angiogenesis (Chen et al., 2012; Hoopes et al., 2015; Rocic and Schwartzman, 2018). This study is the first to uncover the underlying molecular and cellular mechanisms by which neutrophil-derived MPO and HOCl critically contribute to 20-HETE production that drives ischemic angiogenesis via a HIF-1α- and CYP4A11-dependent mechanism. This is based on three major findings.

Firstly, neutrophils critically contribute to increased post-ischemic 20-HETE production and angiogenesis. Neutrophils are quickly recruited to sites of ischemic injury (Mayadas et al., 2014) and their actions in the initial phase of angiogenesis are extensively documented (Noonan et al., 2008; Gong and Koh, 2010). These actions include mobilizing angiogenic factors such as VEGF (Gong and Koh, 2010), and the release of collagenases to ensure the access of these factors to their targets (Christoffersson et al., 2012). Increased plasma 20-HETE is associated with neutrophil activation and endothelial expression of adhesion factors (Joseph et al., 2017) which reinforces the notion that neutrophil-mobilized VEGF can positively feedback with 20-HETE in stimulating ischemic angiogenesis (Chen et al., 2014).

Secondly, neutrophil–derived MPO and HOCl play a crucial role in driving post-ischemic 20-HETE increases and angiogenic responses. The neutrophilic MPO released at angiogenic sites restructures the ECM to accommodate nascent vascular tissues (Rees et al., 2012; DeNichilo et al., 2015). Moreover, MPO-derived oxidants activate angiogenic pathways (Colavitti et al., 2002; Xia et al., 2007; Panagopoulos et al., 2015; Khalil et al., 2018; Vanhamme et al., 2018). In our study, ischemia fails to
induce 20-HETE increases, and post-ischemic angiogenesis is reduced in both MPO\textsuperscript{-/-} and neutrophil-depleted mice. This suggests that these effects are restricted to neutrophils and specifically MPO because ischemia-induced 20-HETE production and angiogenesis remain unaffected in monocyte/macrophage-depleted mice. MPO is most abundantly expressed in neutrophils followed by monocytes (Schultz and Kaminker, 1962; Bos \textit{et al.}, 1978). However, monocytes lose the ability to synthesize and package MPO following differentiation into macrophages and migration into tissues (Klebanoff, 1980). Our data are consistent with neutrophil-derived MPO being mainly responsible for the 20-HETE increases that drives angiogenesis post-ischemia.

The detection of HOCl in biological systems is challenging as it is highly reactive and short-lived (Winterbourn, 2002). The reaction of hypohalous acids with biological molecules forms novel products such as 3-chlorotyrosine: generated by the chlorination of tyrosyl residues (Hazen and Heinecke, 1997) and glutathione sulfonamide (GSA): a unique cyclized analog of glutathione formed following thiol group chlorination (Pullar \textit{et al.}, 2001) which can be detected by mass spectrometry. Chronic inflammation produces detectable amounts of 3-chlorotyrosine and GSA. Another class of novel indicators react with HOCl with exceptional selectivity and sensitivity and allow for real-time detection \textit{in situ}. Of these compounds, we selected FDOCl-1: a near-infrared dye containing a formyl group which reacts specifically with HOCl and not its derivatives or other oxidants and generates a red signal upon excitation (Wei \textit{et al.}, 2018). The conditions capable of activating the signaling cascade we describe are near-physiological and require the sensitivity of these dyes. We successfully optimized the
conditions for imaging using FDOCl-1 and detected HOCl as early as 30 minutes in ischemic hindlimbs.

In our study, post-ischemic blood perfusion recovery was used as a semi-quantitative measurement of angiogenesis. The changes in perfusion we observe following ligation are consistent with femoral artery anatomy and reflect both the collateral circulation and the collective flow from nascent micro vessels. The initial changes in perfusion are derived from collateral vessels which partly obviate the ligation and subsequent changes are predominantly due to the formation of new vessels. Furthermore, the use of perfusion as a metric for angiogenesis confirms that the nascent vessels are functional. However, the perfusion data can be confounded by local changes in blood pressure. This consideration is critical to experiments done in MPO−/− animals since MPO and its products decrease NO bioavailability by inactivation of endothelial nitric oxide synthase (eNOS) and consequently blunt its effects on perfusion (Eiserich et al., 2002; Cheng et al., 2019). Accordingly, we always diligently confirmed with results from MVD analysis to ensure that changes in perfusion reflect changes in vessel density.

Thirdly, MPO and HOCl increase endothelial 20-HETE production via HIF-1 and CYP4A11 in HDMEC. We observed that HOCl rapidly enters HDMEC and increases CYP4A11 expression and 20-HETE production. MPO alone fails to induce CYP4A11. Low fluxes of H₂O₂ oxidize the reactive heme group in MPO from the native ferric to the highly reactive oxy-ferryl porphyrin radical-cation species capable of oxidizing chloride to HOCl (Furthmüller et al., 2006). The absence of CYP4A11 induction by MPO presumably reflects the efficient catabolism of intracellularly produced H₂O₂ under
tissue culture conditions. Thus, we generated a flux of \( \text{H}_2\text{O}_2 \) using glucose oxidase and glucose instead of adding a bolus of this peroxide, which at high concentrations can reduce rather than oxidize MPO’s oxy-ferryl heme (Marquez et al., 1994). Catalytic activation of MPO significantly elevates CYP4A11 mRNA, protein, and 20-HETE, strongly implicating HOCl as a potent stimulator of CYP4A11 expression and 20-HETE production.

HIF is highly sensitive to changes in oxygen tension (Schofield and Ratcliffe, 2004) and its activation in ischemic tissues is a major contributor of angiogenesis (Pugh and Ratcliffe, 2003). However, HIF activation in ischemic tissues precedes activation in hypoxic cell culture (Chen et al., 2014, 2016). Though this may reflect the higher oxygen demand in tissues, the presence of neutrophil-derived oxidants may also contribute. HIF signaling can be triggered by ROS (Chandel et al., 2000; Hagen, 2012). We found that addition of HOCl to HDMEC increases HIF-1\( \alpha \) protein expression and HIF transcriptional activity within 30 min, consistent with HOCl being a potent inducer of HIF signaling independent of hypoxia. We were unable to establish a temporal relationship between HIF and CYP4A11 at timepoints earlier than 30 mins. To circumvent this limitation, we established a causative relationship between HIF and CYP4A11 using two HIF inhibitors with distinct mechanisms of action. Both SCBT and HIFI-V effectively abolish HOCl-induced CYP4A11 mRNA and attenuate CYP4A11 protein expression. Accordingly, we conclude that HOCl-mediated CYP4A11 upregulation requires HIF activation. Even so, HOCl and MPO may promote the release of 20-HETE from HDMEC by the other mechanisms: 1) direct activation of CYP4A11 by oxidation of critical cysteine residues, 2) chlorination of AA can influence its substrate behavior for
CYP4A11 (Brahmbhatt et al., 2010) 3) MPO can act as a hydroxylase and directly hydroxylate AA to 20-HETE (Zhang and Elfarra, 2017) 4) HOCl may stimulate AA production (Korotaeva et al., 2013). Further study is warranted to elucidate these mechanisms.

Thus far, our discussion has focused on the production of HOCl and its consequences on angiogenesis. Although, a portion of HOCl forms unique chlorinated species capable of activating a myriad of signaling events (Midwinter et al., 2001, 2004). Chloramines, which are produced as a result of the reactions between HOCl and amine-bearing groups (Zgliczyński et al., 1971), are amongst these species and will be a major focus for our future studies.

In addition, HIF-1α activation upregulates the production of VEGF, a potent angiogenic factor (Forsythe et al., 1996). Our group previously reported that a positive feedback regulatory loop exists between the CYP4A/20-HETE and VEGF pathway in cultured vascular cells (Chen et al., 2014, 2016, 2019). Thus, the angiogenic activity ascribed to HIF-CYP4A/20-HETE may also be derived from the HIF-mediated production of VEGF and its subsequent feedforward stimulation of 20-HETE production.

Taken together, these findings suggest that ischemia stimulates 20-HETE increases that lead to angiogenesis via a cascade of events (Fig 6). Ischemia (1) leads to the recruitment of neutrophils to the site of injury (2). Neutrophils subsequently de-granulate and release MPO directly to the injured endothelium (2). In the presence of H₂O₂, MPO generates HOCl (3). HOCl enters endothelial cells and induces HIF activity (4), which in turn leads to the increased expression of CYP4A11 (5). CYP4A11
catalyzes the conversion of AA to 20-HETE (6), which triggers post-ischemic compensatory angiogenesis (7).

Since 20-HETE exerts pronounced effects on ischemia-induced angiogenesis, targeting this pathway may be effective for the treatment of pathological angiogenesis (Zuazo-Gaztelu and Casanovas, 2018; Lugano et al., 2019). Moreover, MPO may be involved in pathological 20-HETE production in other systems. As the detrimental effects resulting from the aberrant release and expression of MPO became more evident, the demand for pharmacological inhibitors increased significantly (Forbes et al., 2013; Lazarevic-Pasti et al., 2015; Wurtz et al., 2018; Galijasevic, 2019; Davies, 2021). Synthetic compounds such as 4-Aminobenzoic acid hydrazide (Kettle et al., 1995, 1997) alleviates MPO-induced injury in murine models of atherosclerosis (Tiyerili et al., 2016) and stroke (Forghani et al., 2015), PF-1355 mitigates injury due to myocardial infarction (Ali et al., 2016) and AZM198 alleviates inflammation in atherosclerotic plaques (Rashid et al., 2018) and the vascular dysfunction that arises from pulmonary hypertension (Klinke et al., 2018). MPO inhibitors are currently being tested for therapeutic use in clinical trials and are effective and well-tolerated in patients (Jucaite et al., 2015; Antonelou et al., 2020; Nelander et al., 2020). Thus, the application of pharmacological MPO inhibitors represent a promising approach for the treatment of vascular disease.

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Authorship Contributions

Participated in research design: Azcona, Tang, Falck, Schwartzman, Jeitner, Guo.

Conducted experiments: Azcona, Tang, Berry, Zhang, Garvey.

Contributed new reagents or analytical tools: Falck, Yi, Jeitner.

Performed data analysis: Azcona, Tang, Berry, Garvey, Falck, Schwartzman, Jeitner, Guo

Wrote or contributed to the writing of the manuscript: Azcona, Tang, Schwartzman, Jeitner, Guo


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Guo AM, Scicli G, Sheng J, Falck JC, Edwards PA, Scicli AG. 20-HETE can act as a


Lugano R, Ramachandran M, Dimberg A. Tumor angiogenesis: causes,


Footnotes

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Figure Legends

Figure 1. Neutrophil-derived MPO critically contributes to post-ischemic increases in 20-HETE production and angiogenesis. Femoral artery ligations were performed in Balb/C, Balb/C with neutrophils depleted by treating the animal with 0.5 mg of Ly6G/C antibody (i.p.), Balb/C depleted of macrophages/monocytes with 5 mg/ml Clodronate Liposomes (i.p.), and MPO⁻/⁻ mice (global myeloperoxidase knock-out). A) Flow cytometric quantitation of CD11b⁺Gr-1⁺ circulating neutrophils in blood isolated from untreated control mice and mice treated with Ly6G/C antibodies at days 0, 1-, 3-, 7-, and 14-days post ligation (mean ± SEM; n=4-6; *p<0.05 vs corresponding controls, one-way ANOVA, repeated measures, Dunnett post-hoc). B) Hindlimb gracilis muscles were harvested 3 days post-ligation and homogenized. Non-ischemic muscles were used as controls. 20-HETE was measured by LC/MS/MS and samples were normalized to their corresponding non-ischemic controls (mean ± SEM; n=6-8; *p<0.05 vs corresponding NI control (9.2±1.8 pg/mg protein) and #p<0.05 vs ischemic controls (36.6±4.7 pg/mg protein), one-way ANOVA, Tukey post-hoc); C) LDPI was performed to assess blood perfusion recovery in the ischemic hindlimbs at days 21 post-ligation. Representative blood perfusion scans are shown for control, neutrophil-depleted, and MPO⁻/⁻ mice (mean ± SEM; n=6-8; *p<0.05 vs corresponding NI control and #p<0.05 vs ischemic controls, two-way ANOVA, Tukey post-hoc); D) Quantitation of blood perfusion recovery to ischemic hindlimbs at days 21 (mean ± SEM; n=6-8; *p<0.05 vs corresponding NI control and #p<0.05 vs ischemic controls, one-way ANOVA, Tukey post-hoc); E) Ischemic gracilis muscles were also extracted at days 21 post-ligation and frozen sectioned. Contralateral non-ischemic gracilis muscles were used as controls. Immunofluorescent co-localization staining of tomato lectin (Green) and micro-vessel marker CD31 (Red)
were carried out and the numbers of tomato lectin+CD31+ micro-vessels were counted for MVD analysis. NI: non-ischemic; I: Ischemic. (mean ± SEM; n=6-8; *p<0.05 vs corresponding NI control and #p<0.05 vs ischemic controls, two-way ANOVA, Tukey post-hoc).

**Fig 2.** Ischemia leads to significant MPO deposition and HOCl formation in mouse hindlimb. Mice were again subjected to femoral artery ligation to induce hindlimb ischemia. A) Gracilis muscles from non-ischemic and ischemic hindlimbs were extracted and frozen sectioned 16 hr post-ligation. Muscle samples were incubated with anti-MPO (1:250) antibody overnight, followed by incubation with anti-goat FITC-conjugated secondary antibodies (1:1000) for 1 hr. Immunofluorescent microscopy was performed and representative images of MPO reactivity were shown (n=3). Scale bar=10 µm; B) HOCl formation was determined and quantitated live at 30 min post-ischemia (n = 6) using the Xenogen In-vivo Imaging System (IVIS) Spectrum by injecting the FDOCl-1 HOCl-specific fluorescent probe (1 mM) directly into both hindlimb gracilis muscles for 5 min. In a different group of mice, HOCl (25 μM, n = 6) or LPS (10 μg, 1hr, n = 4) was injected into hindlimb gracilis muscle as positive controls. Mock surgeries were performed on the contralateral hindlimbs with sterile saline injection serving as the non-ischemic solvent control. Total epi fluorescence from each group was determined and quantitated compared to their respective non-ischemic solvent controls. (mean ± SEM; *p < 0.05, paired t-test).

**Fig 3.** HOCl and catalytically active MPO significantly induce CYP4A11 mRNA and protein expression in HDMEC. A) The kinetics of HOCl entry into HDMEC in vitro was determined live by immunofluorescence microscopy in cultures preloaded with FDOCl-1
(10 μM) dye. Hoescht 33342 were used to counter stain for the nuclei. The gradual increase of HOCl influx into the cultures was qualitatively assessed based on fluorescence intensity of FDOCl-1 in response to 10-min HOCl incubation. Representative images of the time dependent HOCl entry into HDMEC are shown (n=3). Another group of HDMEC were incubated with HOCl (15 μM) for 5 min. The effects of HOCl on B) 20-HETE synthase CYP4A11 mRNA expression was assessed by quantitative real-time PCR and C) CYP4A11 protein expression was determined using western blot analysis at 0.5, 1, 2, 4, and 16 hr post-HOCl incubations (mean ± SD; n =3-6; *p<0.05 vs. untreated control, one-way ANOVA, Dunnett post-hoc). Additional HDMEC were also incubated in MPO alone or MPO + GOx + Glucose for 15 min to determine the effects of these treatments on the expression of CYP4A11 mRNA D) and protein E) at time points of maximal CYP4A11 expression for both parameters (1 hr for mRNA, 16 hr for protein). (mean ± SD; n = 3; *p< 0.05 vs. untreated control, one-way ANOVA, Dunnett post-hoc). Finally, F) 20-HETE production in HDMEC exposed to MPO, MPO + GOx, or HOCl was measured as previously described using LC/MS/MS analysis. (mean ± SD; n = 3 in triplicates; *p<0.05 vs. vehicle controls, one-way ANOVA, Dunnett post-hoc).

Figure 4. HOCl upregulates HIF1α protein expression and promotes HIF1α transcriptional activity in HDMEC. HDMEC were incubated with HOCl (15 μM) for 5 min. A) HIF1α protein expression was determined at 0.5,1, 2, and 4 hr following incubations by western blotting (mean ± SD; n = 3 in triplicates; *p<0.05 vs. untreated controls, one-way ANOVA, Dunnett post-hoc); and B) another group of HDMEC were transfected with a lentiviral vector carrying a luciferase reporter adjacent to multiple HIF
responsive element (HRE) transcriptional sites as described in the methods. HIF transcriptional activity was measured by luciferase activity at 0.5, 1, 2, and 4 hr following 5-min HOCl (15 μM) exposure (mean ± SD; n = 3; *p<0.05 vs. untreated control, one-way ANOVA, Dunnett post-hoc). All data was normalized as fold of vehicle controls.

**Figure 5. CYP4A11 induction by HOCl are HIF-dependent.** HDMEC were pre-treated with HIF inhibitors HIFI-V or SCBT for 16 hr (0.5 μM), and then exposed to HOCl (15 μM) for an additional 5 min. DMSO treated cultures were used as the vehicle control. A) Schematic illustration of the differential mechanism of actions of HIF inhibitors HIFI-V and SCBT; B) CYP4A11 mRNA expression was determined 1 hr after exposure to HOCl using quantitative real-time PCR and normalized to their vehicle control (mean ± SD; n = 3; *p<0.05 vs. vehicle controls and #p<0.05 vs. HOCl, one-way ANOVA, Tukey post-hoc); and C) CYP4A11 protein expression was examined and quantitated after 16 hr using western blot analysis. All data was normalized as fold of vehicle controls. (mean ± SD; n = 3-5; *p<0.05 vs. vehicle controls and #p<0.05 vs. HOCl, one-way ANOVA, Tukey post-hoc).

**Figure 6. Schematics by which neutrophil-derived MPO and HOCl contribute to 20-HETE increases that drive post-ischemic angiogenesis.** Ischemic injury (1) such as femoral artery ligation results in recruitment of inflammatory neutrophils (2) to the target vasculature. Neutrophils then release MPO at the site of injured endothelium. MPO subsequently generates HOCl in the presence of H₂O₂ (3). Production of HOCl stimulates the activation of HIF-1α signaling (4), which in turn upregulates the 20-HETE synthase CYP4A11 (5). Consequently, increased CYP4A11 expression leads to
increased endothelial 20-HETE production \((6)\), which drives post-ischemic angiogenesis \((7)\).
Figure 1

A. Gr-1$^+$CD11b$^+$ Neutrophils (x10^6) per ml blood vs. Days Post Ligation

- Control
- anti-Ly6G/C

B. Fold of Non-ischemic Control (20-HETE pg/30 min/mg protein)

- NI
- I
- C.L.
- anti-Ly6G/C
- MPO$^-$

C. Images of Day 1 and Day 21 for control, anti-Ly6G/C, and MPO$^-$

D. Blood Perfusion Ratio (ischemic/non-ischemic)

- Day 1
- Control
- anti-Ly6G/C
- MPO$^-$

E. MVD (Vessel/fiber Ratio)

- NI
- I
- anti-LY6G/C
- MPO$^-$
Figure 2

A. **Non-Ischemic** versus **Ischemic**

B. **25 μM HOCl** versus **10 μg LPS**

Fold of Control (FDOCl-1 Fluorescence)

- **Saline**
- **25 μM HOCl**
- **Saline**
- **10 μg LPS**
- **Non-ischemic**
- **Ischemic**

* indicates significant difference.
Figure 3

A. Image showing fluorescence images of cells at different times (0 min, 2 min, 5 min, 10 min).

B. Bar graph showing relative expression of CYP4A11 mRNA over time (0, 0.5, 1, 2, 4, 16 hours).

C. Western blot showing CYP4A11 (55 kDa) and β-actin (42 kDa) expression over time (0.5, 1, 2, 4, 16 hours).

D. Bar graph showing relative expression of CYP4A11 mRNA with MPO and MPO + GOx treatment.

E. Western blot showing CYP4A11 (55 kDa) and β-actin (42 kDa) expression with MPO and MPO + GOx treatment.

F. Bar graph showing 20-HETE levels over time (0.5, 1, 2, 4, 16 hours) with different treatments.
Figure 4

A. Time (hr)  
Control 0.5 1 2 4  
HIF-1α (90 kDa)  
β-actin (42 kDa)  
Fold of Control (HIF-1α protein expression)  
Control 0.5 1 2 4  
Time (hr)  

B. Time (hr)  
Control 0.5 1 2 4  
Relative HIF-1α Luciferase Activity (luminescence/µg protein)
**Figure 5**

A. **HIF Inhibitor V (HIFI-V)**
((E)-3-(3-(4-Adamantan-1-ylphenoxy)acrylamido) benzoic acid morpholinoethyl ester)

B. **sc205346 (SCBT)**
(methyl 3-[[2-[4-(2-adamantyl)phenoxy]acetyl]amino]-4-hydroxybenzoate)

**Graph B.**

- **Control**
- **HOCI**
- **HOCI + HIFI-V**
- **HOCI + SCBT**

Relative Expression of CYP4A11 mRNA

* * *

# #

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Figure 5

C.

CYP4A11
(55 kDa)

β-actin
(42 kDa)

Fold of Control

Control  HOCl  HOCl+HIFI-V  HOCl+SCBT

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Figure 6
Supplemental Data

**Neutrophil-derived Myeloperoxidase and Hypochlorous Acid Critically Contribute to 20-HETE Increases that Drive Post-Ischemic Angiogenesis**

Running Title: Mechanism of 20-HETE regulation of ischemic angiogenesis

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Supplementary Materials and Methods

Validation of anti-CYP4A11 and anti-HIF-1α Antibodies for Western Blot Analysis

HDMEC were incubated in 5 ml PBS containing 15 μM HOCl for 5 min. Cells were then washed and incubated in serum-free basal media and harvested with RIPA buffer containing protease inhibitor (Sigma – Cat No. 539131) after 1 hr for HIF-1α and 16 hr for CYP4A11, respectively. Lysates were quantified and equal amount of protein lysates (25 μg) were loaded and separated on a 12% tris-glycine gel with a pre-stained protein molecular weight marker (Cat. No-928-60000, Li-CoR, Lincoln, NE). Proteins were transferred to a PVDF membrane overnight and were incubated in anti-CYP4A11 (Sigma-SAB2702082) (1:2500), anti-HIF1α (Abcam-ab16066) (1:500) at 4 °C overnight followed by incubation with anti-rabbit IRDye 800 CW secondary antibody (Cat. No - 926-32211) or anti-mouse IRDye 680 RD secondary antibody (Cat. No - 926-68070) (1:10,000) (LI-COR) at RT for 2 hr. The blots were then scanned with LI-COR Odyssey Infrared Imaging System.

Flow cytometric analysis

Whole blood was collected from untreated control mice and mice treated with Ly6G/C antibody at days 0, 1, 3, and 7 post-ligation. Trucount flow cytometry experiments were performed on 25 μl of total blood directly stained with anti-CD11b (Abcam, Cambridge, MA) and anti-CD115 (BioLegend, San Diego, CA) antibodies washed into 2 ml of PBS then mixed with 25 μl of countBright absolute counting beads (Invitrogen) and resuspended into 2 ml of PBS before acquisition on a LSR II flow cytometer (BD
Biosciences). Live cells were gated based on their forward and side angle light scatter and analyzed using CellQuest Pro software (BD Biosciences). Circulating monocyte quantitation was expressed as CD115⁺CD11b⁺ (x10⁶) cells per ml of blood.

**In vivo live imaging of HOCl**

Femoral artery ligation was performed to introduce ischemia to the right hindlimb of MPO⁻/⁻ mice. A mock surgery was carried out on the contralateral left hindlimb as the non-ischemic control. Detection of HOCl in hindlimb tissues was assessed and quantitated by subsequently injecting a HOCl-specific fluorescent probe: FDOCl-1 (Wei et al., 2018) (10 μL; 1 mM) into both hindlimbs. Fluorescence generated by the FDOCl-1 deformylation product was then detected 5 min after FDOCl-1 injections using the Xenogen In-vivo Imaging System (IVIS) Spectrum from Caliper Life Sciences (λ_em = 720nm, λ_ex = 640nm). All images were captured using the Living Image Software (IVIS Imaging Systems, Shelton, CT) under settings recommended by the manufacturer.

**Real-time PCR of CYP4A11 Transcript**

HDMEC were first incubated in GOx (50 μg/mL) + Glucose (250 μM) for 15 minutes. Cells were then incubated in serum-free basal media for 1 hr and harvested with RLT Lysis Buffer (Qiagen, Hilden, Germany) containing 1% BME and RNA was isolated using the RNeasy extraction kit (Qiagen). RNA was quantified using Take 3 Micro-Volume Plate and 500 ng of RNA was loaded for cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). Newly synthesized cDNA was loaded onto a 96-well PCR plate and incubated with Taqman Master Mix (Thermo
Fisher Scientific) and the FAM-MGB probes (assay IDs shown in parentheses): CYP4A11 (Hs04194779_g1; Thermo Fisher Scientific) and GAPDH (Hs02786624_g1; Thermo Fisher Scientific). RT-PCR was performed using the QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA) using the following conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min, and 50°C for 2 min. The threshold cycle (Ct) data was determined using default threshold settings. The Ct was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative changes in the gene expression.

*Western Blot Analysis of CYP4A11*

HDMEC were incubated in GOx (50 μg/mL) + Glucose (250 μM) for 15 min. Cells were then incubated in serum-free basal media and harvested with RIPA buffer containing protease inhibitor (Sigma – Cat No. 539131) after 16 hr. Lysates were quantified and equal amount of protein lysates (25 μg) were loaded and separated on a 12% tris-glycine gel. Proteins were transferred to a PVDF membrane using a methanol-glycine-tris base buffer overnight. Next, membranes were incubated in anti-CYP4A11 (Sigma-SAB2702082) (1:2500) at 4 °C overnight followed by incubation with anti-rabbit IRDye 800 CW secondary antibody (Cat. No - 926-32211) at RT for 2 hr. The blots were then scanned with LI-COR Odyssey Infrared Imaging System. β-actin (Santa Cruz Biotechnology SC47778) (1:10,000) was used as a loading control. The blot was quantitated using NIH ImageJ software by comparing the density ratios of control band/β-actin to experimental band/β-actin.
in vitro MPO and HOCl Studies

HDMEC were incubated in GOx (50 µg) and various concentrations of glucose (100 µM, 250 µM, 500 µM) in a 1 ml reaction mix for a period of 15 min. A modified FOX method were used to determine H$_2$O$_2$ concentrations. FOX reagent composed of 200 µM xylenol orange (Sigma), 200 mM sorbitol (Sigma), and 25mM H$_2$SO$_4$ was adjusted to a pH of 1.75 by the addition of dibasic sodium phosphate and was prepared freshly by addition of 500 µM ferrous ammonium sulfate (Sigma). H$_2$O$_2$ concentrations were determined by mixing 100 µL of unknown samples with 900 µL of working reagent. These samples were incubated at RT for 30 minutes for color development, centrifuged at 10,000 x g for 5 minutes, and aliquoted to a 96-well plate (200 µl per sample) where absorbance was measured at 560 nm. We developed an assay for the determination of HOCl concentrations in the presence of MPO (1.25 µg), GOx (50 µg), and glucose, which was carried out in 1 ml reaction volumes (1x PBS) containing 10 µM FDOCl-1. Known concentrations of HOCl were made and pre-determined both by TNB and absorbance at 292 nm and used to create a standard curve for detection by FDOCl-1. HOCl concentrations were subsequently detected in enzyme mixtures by absorbance of the FDOCl-1 deformylation product at 668 nm. This assay reliably measures HOCl concentrations in the 5-30 µM range.
Supplementary Figures and Figure Legends

*Validation of HIF-1α and CYP4A11 antibodies.*

A commercially purchased standard protein weight ladder was used to individually validate the anti-HIF-1α and anti-CYP4A11 antibodies we used in our studies. Lysate from HDMEC treated with HOCl (15 μM for 5 min) were harvested after 1 hr for HIF-1α and 16 hr for CYP4A11, respectively. Western blot analysis was performed as previously described. We showed that positive HIF-1α reactivity occurred at ~90 kDa and CYP4A11 antibody reactivity at ~55 kDa, consistent with the manufacturers’ recommendations (Supp. Fig. 1).

**Supplementary Figure 1.** Validation of HIF-1α and CYP4A11 antibodies using protein standard molecular weight marker.
Effects of anti-Ly6G/C antibody on circulating monocytes

Anti-Ly6G/C antibody can impose off-target depletion effects on circulating monocytes. We performed flow cytometric quantitation of CD115^+CD11b^+ monocyte from the peripheral blood of Balb/C mice treated with/without anti-Ly6G/C antibody. Data in Supp. Fig. 2 shows that anti-Ly6G/C also led to a modest reduction in circulating monocytes (~20%).

Supplementary Figure 2. Effects of anti-Ly6G/C on circulating monocytes. Femoral artery ligations were performed as described. Flow cytometric quantitation of CD115^+CD11b^+ circulating monocytes in blood isolated from untreated control mice and mice treated with anti-LY6G/C antibodies (0.5 mg) at days 0, 1-, 3-, and 7-days post ligation (mean ± SEM; n=4; *p<0.05 vs corresponding controls, one-way ANOVA, repeated measures, Dunnett post-hoc).
Detection of HOCl in MPO−/− mice post-ischemia.

In Figure 2B, we demonstrated that HOCl is produced in the ischemic hindlimbs as indicated by fluorescence of the FDOCl-1 deformylation product. To confirm that HOCl is produced endogenously by MPO in these tissues, we performed the same experiment in MPO−/− mice. As we expected, FDOCl-1 fluorescence was not detected in either limb, suggesting that ischemia fails to induce HOCl production in the absence of MPO (Supp. Fig. 3).

Supplementary Figure 3. In vivo HOCl detection in MPO−/− mice. Femoral artery ligation was performed in the right hindlimb of MPO−/− mice. Mock surgeries were performed on the contralateral left limbs as the non-ischemic controls. HOCl formation was determined and quantitated live at 30 min post-ischemia (n = 3) using the Xenogen In-vivo Imaging System (IVIS) Spectrum by injecting the FDOCl-1 fluorescent probe (1 mM) directly into both hindlimb gracilis muscles and imaging after 5 min.
Effects of Glucose and Glucose oxidase on CYP4A11 transcripts and protein expression in HDMEC.

To closely mimic the conditions under which HOCl is generated by MPO in vivo, we opted to incubate HDMEC in pre-optimized concentrations of glucose, glucose oxidase, and MPO. This approach allows us to analyze the effects of these compounds in concert and in isolation. We determined the effects of basal H$_2$O$_2$ generated by glucose/glucose oxidase alone on CYP4A11. Though glucose oxidase/glucose elevates CYP4A11 transcript (1.46-fold ± 0.18) and protein (1.56-fold ± 0.10) (Supp. Fig. 4A&B), these changes are significantly reduced compared to the changes induced by HOCl generated by MPO (transcript: 2.86-fold ± 0.26, protein: 2.24-fold ± 0.56). Since a significant portion of H$_2$O$_2$ is consumed by the peroxidase activity of MPO, the H$_2$O$_2$ concentrations necessary for the induction of CYP4A11 may not actually be present. Thus, we infer that HOCl generated by MPO is the major factor for the induction of CYP4A11 and 20-HETE production in our experimental setting.
Supplementary Figure 4. Induction of CYP4A11 Transcript and Protein in Response to H₂O₂ Generated by Glucose Oxidase and Glucose in the Absence of Myeloperoxidase. HDMEC were incubated in GOx (50 µg/mL) + glucose (250 µM) for 15 minutes and CYP4A11 (A) mRNA (mean ± SD; n = 3; *p < 0.05 vs. untreated control, unpaired t-test) and (B) protein (mean ± SD; n = 3; *p < 0.05 vs. untreated control, unpaired t-test) expressions were determined at time points of maximal CYP4A11 expression for both parameters (1 hr for mRNA, 16 hr for protein).
Determination of $H_2O_2$ and HOCl concentrations generated by the glucose/GOx/MPO system.

To ensure the reproducible generation of 15 µM HOCl, we first optimized the individual concentrations of glucose, GOx, and MPO before incubating HDMEC in a reaction mixture containing glucose/GOx/MPO. Initially, we determined the concentrations of glucose and GOx needed to produce $H_2O_2$. GOx at a concentration of 50 µg/ml incubated in 250 µM glucose sufficiently generated 15-20 µM $H_2O_2$ over 15 min (Supp. Fig. 5A). We then established a linear relationship between known HOCl concentrations (as measured by TNB and optical density at 292 nm) and absorbance of the FDOCl-1 deformylation product (668 nm) (Supp. Fig. 5B). Next, we ascertained the concentrations of HOCl generated by glucose/GOx/MPO in the presence of FDOCl-1. These concentrations were determined in reaction mixtures containing MPO (1.25 µg/mL), GOx (50 µg/ml), and different concentrations of glucose (250 µM, 300 µM, 350 µM). (Supp. Fig. 5C). Although 350 µM glucose effectively produces 15 µM HOCl under these conditions, we observed a significant amount of cytotoxicity in our HDMEC cultures. Hence, we reduced the glucose concentration to 250 µM which generates 10 µM HOCl. Toxicity was not observed under these conditions.
Supplementary Figure 5. Generation of H₂O₂ and HOCl by the Glucose/Gox/MPO system. (A) Concentrations of H₂O₂ generated at 1, 2, 4, 8, and 15 min by 50 µg of GOx in a 1 ml reaction mix containing 250, 300, or 350 µM glucose (mean ± SD; n = 3). (B) Absorbance of FDOCl-1 (10 µM) deformylation product (686 nm) in the presence of pre-determined concentrations of HOCl (0-25 µM) (mean ± SD; n = 4). (C) Concentrations of HOCl generated at 1, 2, 4, 8, and 15 min by 50 µg GOx and 1.25 µg MPO in a 1 ml reaction mix containing 250, 300, or 350 µM glucose (mean ± SD; n = 3).