Reactive Oxygen Species Regulate Osteopontin Expression in a Murine Model of Postischemic Neovascularization


Objective—Previous findings from our laboratory demonstrated that neovascularization was impaired in osteopontin (OPN) knockout animals. However, the mechanisms responsible for the regulation of OPN expression in the setting of ischemia remain undefined. Therefore, we sought to determine whether OPN is upregulated in response to ischemia and hypothesized that hydrogen peroxide (H$_2$O$_2$) is a critical component of the signaling mechanism by which OPN expression is upregulated in response to ischemia in vivo.

Methods and Results—To determine whether ischemic injury upregulates OPN, we used a murine model of hindlimb ischemia. Femoral artery ligation in C57BL/6 mice significantly increased OPN expression and H$_2$O$_2$ production. Infusion of C57BL/6 mice with polyethylene glycol-catalase (10 000 U/kg per day) or the use of transgenic mice with smooth muscle cell-specific catalase overexpression blunted ischemia-induced OPN, suggesting ischemia-induced OPN expression is H$_2$O$_2$-dependent. Decreased H$_2$O$_2$-mediated OPN blunted reperfusion and collateral formation in vivo. In contrast, the overexpression of OPN using lentivirus restored neovascularization.

Conclusion—Scavenging H$_2$O$_2$ blocks ischemia-induced OPN expression, providing evidence that ischemia-induced OPN expression is H$_2$O$_2$ dependent. Decreased OPN expression impaired neovascularization, whereas overexpression of OPN increased angiogenesis, supporting our hypothesis that OPN is a critical mediator of postischemic neovascularization and a potential novel therapeutic target for inducing new vessel growth. (Arterioscler Thromb Vasc Biol. 2012;32:1383-1391.)

Key Words: osteopontin ▪ reactive oxygen species ▪ ischemia ▪ angiogenesis ▪ collateral circulation

The occlusion of blood vessels ultimately leads to ischemia, initiating multiple processes that promote neovascularization as a compensatory mechanism to restore blood flow and preserve tissue function. The ability to develop new collaterals is strongly associated with reduced long-term cardiac mortality in patients with acute myocardial infarction and stable coronary artery disease. The formation of new collaterals is a multifactorial process that requires cytokines, the infiltration of inflammatory cells, cell proliferation, cell migration, and matrix remodeling. Several of these processes are modulated by the presence of osteopontin (OPN), a secreted phosphorylated matricellular protein critical for neovascularization.

OPN is important for normal arterial physiology and is expressed by multiple cell types including monocytes/macrophages, endothelial cells, and smooth muscle cells (SMCs), all of which play a role in the neovascularization process. OPN mediates several processes relevant to collateral formation, including cell survival, cell adhesion, and migration. In addition, wound healing, a process that requires angiogenesis, is significantly impaired in OPN−/− mice strongly supporting a role for OPN in angiogenesis. Moreover, our group previously demonstrated a direct role for OPN in posts ischemic neovascularization by showing dramatically impaired collateral formation in OPN−/− mice compared with wild-type (WT) controls in a murine model of hindlimb ischemia (HLI).

OPN is a noncollagenous, phosphorylated glycoprotein thought to mediate cellular functions by providing a link between cell-surface receptors and structural extracellular matrix molecules. OPN functions in a variety of biological processes and signals by binding to cell-surface integrins through a conserved arginine-glycine-aspartate domain or the SVVYGLR binding domain, which is exposed when OPN is cleaved by thrombin or matrix metalloproteinases. OPN is also a ligand for the CD44 receptor. In addition, OPN promotes the migration of multiple cell types, including macrophages, endothelial cells, and vascular SMCs. An alternative translation start site in the OPN messenger RNA generates 2 OPN isoforms: a secreted form and an intracellular form. Both secreted and intracellular OPN are linked to cell migration, where intracellular OPN localizes to the cell membrane and associates with CD44 and secreted OPN regulates cell responses through association with integrin receptors and the CD44 receptor.

Reactive oxygen species (ROS), such as superoxide (O$_2$•−) and hydrogen peroxide (H$_2$O$_2$), are involved in physiological...
and pathophysiological responses. When produced in excess, ROS promote cell injury and disease pathologies. In contrast, at physiological levels, ROS function as second messengers and regulate redox signaling pathways that modulate cell migration, proliferation, and matrix remodeling, all of which are necessary events for neovascularization.19–22 A recent study by Tojo et al23 provided a direct link between NADPH oxidases and postischemic neovascularization in a murine model of HLI. The study demonstrated that animals lacking gp91phox, a subunit of the Nox2 NADPH oxidase, produce less ROS and form fewer collaterals compared with WT animals.

Although several recent publications link ROS to OPN expression in vitro,24–26 little is known regarding the role of ROS in regulating OPN expression in vivo. Furthermore, the precise molecular identity of the relevant ROS remains unclear. The goals of this study were to determine whether OPN is upregulated in response to ischemia, whether increased ROS production is the mechanism by which OPN expression is increased, and to define the molecular identity of the relevant ROS involved in OPN expression in vivo. In this study, we demonstrate that OPN is upregulated in response to ischemia using a murine model of HLI. Femoral artery ligation in C57BL/6 mice significantly increased OPN expression and H2O2 production in the ischemic limb (IL), compared with the nonischemic limb (NIL). Using pharmacological and transgenic approaches to scavenge H2O2, we demonstrate that ischemia-induced OPN expression is H2O2-dependent. Infusion of mice with polyethylene glycol (PEG)-catalase (10 000 U/kg per day) or the use of transgenic mice with SMC-specific catalase overexpression (TgSMC-Cat) significantly blunted ischemia-induced OPN expression, resulting in delayed reperfusion and impaired collateral formation in response to ischemia. In addition, using the same model of TgSMC-Cat mice, when we used lentiviral delivery to overexpress OPN in the ischemic limb, we were able to completely restore neovascularization in the ischemic limb. Altogether, the results presented herein define a novel mechanism for the regulation of OPN expression in vivo, establish an absolute requirement for H2O2-dependent OPN for effective collateral formation, and support the concept that OPN is a critical mediator of neovascularization. Therefore, OPN may be a novel therapeutic target for promoting collateral vessel formation.

Materials and Methods
A detailed, expanded Materials and Methods section is available in the online-only Data Supplement.

Animals
Male C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). TgSMC-Cat mice were bred in-house in the Department of Animal Resources at Emory University. TgSMC-Cat mice have increased expression of human catalase through the SMC-specific smooth muscle myosin heavy chain promoter and were characterized previously.27 For all experiments TgSMC-Cat mice were compared with WT littersmates. In some experiments, PEG-catalase (10 000 U/kg per day) dissolved in saline was delivered by intravenous infusion via osmotic minipump. PEG-catalase was infused continuously from time of HLI surgery until the designated time points. All animals used were male and between 8 and 10 weeks of age. The animals were housed and cared for according to the guidelines approved by the Emory University Institutional Animal Care and Use Committee.

Osmotic Minipump Implantation
Mice were anesthetized using 3% isoflurane (oxygen delivered at 0.5 L/min with 3% isoflurane for induction and 2.0% isoflurane for maintenance). A catheter attached to a primed osmotic minipump (Alzet osmotic minipump, model 1007D; Durect Corporation, Cupertino, CA) was inserted into the jugular vein and the pump inserted subcutaneously. Mice were administered buprenex (0.01–0.1 mg/kg, SC) as needed.

HLI Surgery
Mice were anesthetized with 3% isoflurane in a chamber and then anesthetized with 2% isoflurane through a nose cone. Ligation and excision of the left superficial femoral artery were performed as described previously.24

Laser Doppler Perfusion Imaging
Laser Doppler perfusion imaging (LDPI) was performed as described previously.3,28 Briefly, mice were anesthetized by inhalation of 2% isoflurane and scanned with the LDPI system (PIM II Laser Doppler Perfusion Imager). Perfusion of the proximal region was quantified and normalized to the nonsurgical limb.

Micro-Computed Tomography Imaging
Quantitative micro-computed tomography (CT) was used for the evaluation of collateral vessel formation in the ischemic limb on postoperative day 5 as described previously.5,28 Briefly, mice were euthanized (n=6 to 9 for each group) and sequentially perfused with saline containing 4 mg/mL of papaverine, 10% formalin, followed by a lead chromate–based contrast agent (Flow Tech, Inc, South Windsor, CT). Bone was demineralized in a formic acid–based solution (Cal-Ex II, Fisher Scientific, Pittsburgh, PA) for 48 hours. Samples were then imaged at a 30-μm voxel size, and the tomograms were used to render binary 3-dimensional images. Stereological algorithms were used to quantify vascular volume to tissue volume ratio, connectivity, and vascular density, which were then normalized to the contralateral control limb.

Lentivirus
The lentiviral vector was derived from the HIV-based lentivirus backbone pLV-CMV-GFP-U3Nhe, as described previously.29–31 pLV-CMV-GFP-U3Nhe control vector will hereafter be referred to as lentivirus (LV)-green fluorescent protein (GFP). We generated a dual-tagged human OPN construct with an HA-tag at the N-terminus and myc-tag at the C-terminus. We inserted a WT internal ribosome entry site,32 followed by dual-tagged OPN, downstream of GFP to generate a lentivirus that expresses both GFP and tagged OPN, hereafter referred to as LV-OPN. The WT internal ribosome entry site allows high translation of the downstream compared with the upstream coding sequence in the same mRNA,32 thereby allowing the final construct to express high OPN and relatively lower GFP protein.

Virality production procedures have been described in detail previously.29,33,34 For in vivo use, both lentiviruses were used at a final concentration of ~1×106 infectious particles/mL. All animals received HLI, after which the IL adductor was injected with 20 μL of LV-GFP or LV-OPN using a similar approach as described previously.28

Detection of ROS
The production of O2·− was evaluated by measuring the conversion of dihydroethidium to 2-hydroxyethidium using high-performance liquid chromatography, as described previously.35 Superoxide production was normalized to protein concentration. H2O2 production was measured using the Amplex Red assay (Invitrogen, Carlsbad, CA),
as described previously.35 H2O2 production was normalized to tissue wet weight.

Immunofluorescence
Mice were euthanized and tissues were perfused with saline and fixed with 10% buffered formalin. Sections from paraffin-embedded hindlimbs were cut into 5 μm increments. Antigen retrieval was performed in citrate buffer, pH 6.0 (Invitrogen), before incubation with OPN antibody (1:200 in 3% BSA), followed by incubation with antimouse or antigoat secondary antibody (1:400 in 3% BSA; Vector Labs, Burlingame, CA) and incubation with Streptavidin QDot 655 (1:200 in 3% BSA; Invitrogen). Images were acquired from a Zeiss Axioskop microscope equipped with an AxioCam camera.

Immunoblotting
Adductor muscle tissues and cultured SMCs were lysed in Hunter buffer.33 Briefly, tissues were homogenized with glass mortar and pestle, and cell samples were sonicated at 10 W for 10 seconds. Whole tissue or cell lysates were used for immunoblotting. The OPN antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and R&D Systems (Minneapolis, MN). GAPDH antibody was also obtained from Santa Cruz Biotechnology. The β-actin antibody was from Cell Signaling (Danvers, MA). Band intensity was quantified by densitometry using ImageJ 1.38 software and expressed normalized to β-actin or GAPDH.

Cell Culture
Murine aortic SMCs (passages 4–10) were cultured in low-glucose DMEM (Sigma Aldrich, Saint Louis, MO) supplemented with 10% FBS (Sigma Aldrich), 2 mmol/L L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin, all acquired from Mediatech, Inc. (Manassas, VA). Cells were stimulated with H2O2 (Sigma Aldrich) after 48 hours of quiescence in serum-free DMEM for all experiments.

RNA Isolation and Quantitative Real-time Polymerase Chain Reaction
Total RNA was extracted from muscle tissue or cells using the RNeasy kit (Qiagen, Valencia, CA). OPN and 18S rRNA, were measured by amplification of cDNA using the thermocycler (Applied Biosystems, Foster City, CA) and SYBR green dye. Copy number was calculated by the instrument software from standard curves of genuine templates. OPN copy number was normalized to 18S rRNA.

Statistical Analysis
Results are expressed as mean±SEM from at least 3 independent experiments. Statistical significance for quantitative results was assessed using ANOVA, followed by Bonferroni multiple comparison post hoc test. In some cases, a Student t test was used to assess significance. A value of P<0.05 was considered statistically significant.

Results
Effects of HLI on OPN Expression
Our previous study demonstrated that OPN−/− mice exhibit impaired collateral formation.3 Therefore, we set out to determine whether OPN is upregulated in response to ischemia by using a murine model of HLI in which the femoral artery was ligated and excised, thus providing a stimulus for collateral formation. We measured OPN protein expression in the adductor muscles of the IL and NIL at 0, 3, 5, 7, 14, and 21 days post-HLI. As shown in Figure 1A, full-length OPN protein expression in the IL peaked between 5 and 7 days post-HLI, followed by a significant decline thereafter. Therefore, we investigated the effects of HLI on OPN expression at 5 days postsurgery. To evaluate whether ischemia increases OPN mRNA levels, we used quantitative real-time polymerase chain reaction (qRT-PCR) to measure OPN message in the adductor muscles of the IL and NIL after 5 days. OPN mRNA levels were increased 20-fold in the IL compared with NIL (Figure 1B). OPN protein expression in the IL was also significantly increased compared with NIL, as measured by Western blot (Figure 1C) and immunofluorescence (Figure 1D).

The ischemia-induced increases in OPN protein appeared as multiple bands by Western blot, presumably because of post-translational modification. Full-length OPN is a complex matricellular protein that is glycosylated, phosphorylated, and cleaved by other proteins after secretion into the extracellular space.36 We detected an increase in both full-length, modified OPN (upper bands in Figure 1C), as well as cleaved OPN (lower band in Figure 1C).

Multiple cell types involved in the process of collateral formation are reported to express OPN in other systems. To determine the specific cell types that contribute to ischemia-induced increases in OPN expression at 5 days posts ischemia, we performed a series of immunofluorescence co staining experiments. As shown in Figure 1D and 1E, multiple cell types express OPN in response to ischemia. OPN clearly colocalizes with Mac3 (Figure 1D), a marker of macrophages, with lectin (Figure 1E), a marker of endothelial cells, and with smooth muscle α-actin (Figure 1F), a marker of SMCs. Taken together, these data demonstrate that ischemia increases OPN expression at the mRNA and protein levels, and that multiple cell types, including macrophages, endothelial cells, and SMCs, contribute to ischemia-induced increases in OPN expression.

Effects of HLI on ROS Production
To determine which ROS, if any, are increased in response to ischemic injury and to establish a time course for ROS production in this model, we used dihydroethidium high-performance liquid chromatography and the Amplex Red assay to measure O2•− and H2O2 production, respectively. ROS were measured in the adductor muscles of the NIL and IL of C57BL/6 mice on postoperative days 3, 5, and 7. There were no detectable differences in O2•− between the IL and NIL at any time point (Figure 2A). In contrast, H2O2 production was significantly increased in response to ischemia at all time points (Figure 2B), with a peak in H2O2 at 5 days.

H2O2 Stimulates OPN Expression In Vivo
As we have demonstrated that in response to ischemia there is a significant increase in H2O2 and that OPN levels are increased in response to ischemia, we set out to determine whether the increases in OPN are H2O2-dependent. We performed HLI on C57BL/6 mice infused with saline or PEG-catalase (10 000 U/kg per day), which converts H2O2 to water and other metabolites, thus diminishing H2O2. PEG-catalase blunted ischemia-induced H2O2 production compared with saline-infused animals (Figure 3A), as measured by Amplex Red assay. This decrease in H2O2 production in the IL substantially blocked ischemia-induced OPN expression at the mRNA (Figure 3B). In addition, we found a similar corroborating result at the
protein level when measured by Western blot and immunofluorescence (Figure 3C, D). Taken together, these data support our hypothesis that the increase in OPN expression in response to ischemia is H$_2$O$_2$-dependent.

Multiple cell types involved in ischemia-induced collateral formation express OPN in response to ischemia, including SMCs (Figure 1F). SMCs are also known to generate ROS and play a vital role in arteriogenesis. To confirm and further substantiate that ischemia-induced OPN expression is H$_2$O$_2$-dependent, and to verify that SMCs may be a substantial contributor to H$_2$O$_2$-dependent OPN expression in the ischemic hindlimb, we used transgenic mice that overexpress catalase specifically in their SMCs (Tg$^{SMC-Ca}$). We then measured ROS and OPN expression in these mice. We found that Tg$^{SMC-Ca}$ mice have significantly diminished ischemia-induced H$_2$O$_2$ production compared with WT controls (Figure 4A). This decrease in H$_2$O$_2$ production mediated by SMC-specific catalase overexpression blunted ischemia-induced increases in OPN mRNA (Figure 4B). In addition, OPN protein levels were substantially blunted in Tg$^{SMC-Ca}$ animals compared with...
ROS-dependent OPN and Angiogenesis

Figure 2. Hindlimb ischemia increases reactive oxygen species (ROS) in the proximal region of the ischemic hindlimb. ROS production was assessed in the adductor muscles of the nonischemic (NIL) and ischemic limbs (IL) of C57BL/6 mice at postoperative days 3, 5, and 7. A, Superoxide production was measured using dihydroethidium high-performance liquid chromatography, where the amount of 2-hydroxyethidium (Oxy-E) was quantified as a readout for superoxide production and Oxy-E signal was normalized (NS; n=5–6). B, The Amplex Red Assay was used to measure hydrogen peroxide (H2O2) levels. H2O2 measurements were normalized to tissue wet weight. H2O2 was increased in the IL compared with the NIL at 3, 5, and 7 days (*P<0.05 vs 3 days). n=3–6. Bars are means±SEM. WT indicates wild type.

WT controls, as measured by Western blot and immunofluorescence (Figure 4C, D). Taken together, these data support that ischemia-induced OPN expression is H2O2-dependent and that SMC-derived H2O2 significantly contributes to ROS-dependent OPN expression.

H2O2-dependent OPN Mediates Collateral Formation

To determine the functional importance of H2O2 and OPN on collateral formation, we used LDPI to evaluate reperfusion and micro-CT to quantify collateral formation in the IL. LDPI images acquired at postoperative day 5 clearly illustrate that PEG-catalase infusion, which decreased ischemia-induced OPN expression and H2O2 levels, impaired reperfusion (Figure IIA in the online-only Data Supplement). Quantitative analysis illustrates a significant lag in perfusion recovery in the IL of PEG-catalase–infused animals (Figure IIB in the online-only Data Supplement). Quantitative analysis of micro-CT images (Figure IIC in the online-only Data Supplement) revealed that PEG-catalase animals have a 19.4% reduction in vascular volume (saline 1.3±0.1 versus PEG-catalase 1.0±0.1; normalized to tissue volume), 8.1% decrease in vascular density (saline 1.2±0.03 versus PEG-catalase 1.0±0.02), and a 41.0% reduction in connectivity (saline 2.6±0.40 versus PEG-catalase 1.5±0.2) compared with controls. Together, these data demonstrate that H2O2 increases OPN expression, which has a direct effect on the formation of collateral vessels after HLI injury.

To determine the functional importance of SMC-derived H2O2 and OPN on collateral formation, we used LDPI to evaluate reperfusion and micro-CT to quantify collateral formation in the IL of TgSMC-Cat animals using LDPI. TgSMC-Cat animals showed significantly decreased reperfusion compared with WT animals (Figure 5A,B). Quantitative analysis of micro-CT images (Figure 5C) revealed TgSMC-Cat mice have a 34.6% reduction in vascular volume (WT 1.23±0.16 versus TgSMC-Cat 0.81±0.06), 14.2% decrease in vascular density (WT 1.04±0.04 versus TgSMC-Cat 0.89±0.04), and a 40.9% reduction in connectivity (WT 1.45±0.20 versus TgSMC-Cat 0.85±0.09) compared with controls. Altogether, these data suggest that SMC-derived H2O2 significantly contributes to ROS-dependent OPN expression and neovascularization.

ROS influence the expression of several inflammatory factors in this model, including monocyte chemoattractant protein-1 (Figure I in the online-only Data Supplement), tumor necrosis factor-α (Figure I in the online-only Data Supplement), and OPN (Figures 3 and 4). Therefore, to determine the relative contribution of OPN to collateral formation compared with other targets downstream of H2O2, we designed an experiment to add OPN back to the ischemic limb. To do this, we developed a lentivirus to overexpress Myc-tagged OPN (LV-OPN). We then used this lentivirus to perform an add-back experiment to determine what effects observed in our TgSMC-Cat model were specifically because of the loss of H2O2-dependent OPN expression versus the loss of other H2O2-dependent proteins. We directly injected the adductor muscles of the IL with either control lentivirus (LV-GFP) or LV-OPN. We verified successful transduction of cells in the adductor muscle by immunofluorescence staining for Myc or GFP (Figure III in the online-only Data Supplement). As expected, the TgSMC-Cat+LV-GFP treatment group had less reperfusion than the WT+LV-GFP group, such as that observed in Figure 5A. Interestingly, the add-back of OPN to TgSMC-Cat animals using LV-OPN (TgSMC-Cat+LV-OPN) restored collateral formation and reperfusion to a level similar to that seen in WT+LV-GFP control animals (Figure 5D, E). These data strongly support OPN as a critical mediator of postischemic neovascularization.

H2O2 Increases OPN Expression In Vitro

Our data demonstrate that ischemia-induced OPN expression in a murine model of HLI is H2O2-dependent and that SMC-derived H2O2 promotes increased OPN expression in the IL. To determine whether H2O2 directly mediates an increase in OPN expression, we used an in vitro system in which mouse aortic smooth muscle cells were quiesced for 48 hours before stimulation with 100 μmol/L H2O2 for 4 hours. Stimulation of mouse aortic smooth muscle cells with H2O2 significantly increased OPN mRNA expression (Figure 6A), cellular OPN protein expression (Figure 6B), and secreted OPN protein expression (Figure 6C) compared with control. Taken
together, these data demonstrate that OPN expression can be directly induced by H$_2$O$_2$ in vitro.

**Discussion**

In this study, we demonstrate that ischemia-induced OPN is H$_2$O$_2$-dependent and is necessary to promote neovascularization. We not only demonstrate that OPN expression in the IL is increased, but that the removal of H$_2$O$_2$ via pharmacological or transgenic approaches significantly blunts H$_2$O$_2$-dependent OPN expression in vivo and results in substantially impaired reperfusion and collateral formation. Our results define a novel mechanism for the regulation of OPN expression by H$_2$O$_2$, demonstrate that H$_2$O$_2$-dependent OPN expression is a critical mediator of postnatal neovascularization, and implicate OPN as a potential therapeutic target for modulating collateral vessel formation.

A recent study provided a direct link between NADPH oxidases, which produce ROS, and postischemic neovascularization in a murine model of HLI. However, the role of specific ROS species, as well as the major cellular sources of ROS, remains unclear. Superoxide has been implicated in the process of neovascularization, where the contributions of O$_2^•$ as a positive or negative contributor to the neovascularization process depends on the overall level of oxidative stress. The role of H$_2$O$_2$ in angiogenesis has been studied in vitro, where it induces the formation of tube-like structures by endothelial cells and
promotes the proliferation and migration of endothelial cells and SMCs. However, a role for H$_2$O$_2$ in the process of collateral vessel formation is yet to be established. Our data show that at 5 days post-HLI, H$_2$O$_2$ production in the IL of C57BL/6 animals was significantly increased (Figure 2B). In contrast, no significant increases in O$_2$•$^-$ were detected in response to ischemia (Figure 2A). Our data clearly implicate a central role for H$_2$O$_2$ in the neovascularization process, where both PEG-catalase–infused mice and Tg$^{SMC-Cat}$ animals, which have decreased H$_2$O$_2$ production (Figures 3A and 4A), exhibit impaired blood flow and neovascularization in response to ischemia (Figure IIA in the online-only Data Supplement and Figure 5A). A recent study from our laboratory established myeloid lineage cells as 1 source of H$_2$O$_2$ in the collateral formation process. The new data presented herein provide evidence that SMC-derived H$_2$O$_2$ promotes neovascularization, as demonstrated by the impaired reperfusion and collateral formation seen in Tg$^{SMC-Cat}$ mice (Figure 5A–C). These data provide strong evidence that neovascularization in response to ischemia is attributable to the effects of H$_2$O$_2$.

OPN is expressed by multiple cell types including monocytes and macrophages, endothelial cells, and SMCs, all of which play a role in the neovascularization process. OPN mediates several processes relevant to collateral formation, including cell survival, cell adhesion, and migration. In addition, our laboratory has previously established OPN as a critical component of the neovascularization process, where OPN$^{-/-}$ mice exhibit dramatically impaired collateral formation in response to ischemia. Our new data show a strong upregulation of OPN in response to ischemia (Figure 1). The increase in OPN mRNA levels in response to ischemia at 5 days was >20-fold. Increased OPN mRNA also translated into substantial increases in OPN protein (Figure 1).

How OPN functions to increase collateral formation remains largely unknown. One mechanism by which OPN may function to promote collateral formation is through its arginine-glycine-aspartate domain.

**Figure 5.** Hydrogen peroxide (H$_2$O$_2$)-dependent osteopontin (OPN) mediates collateral formation. Laser Doppler Perfusion Imaging (LDPI) was used to evaluate perfusion and micro-computed tomography (CT) to quantify collateral formation in the ischemic limb (IL). Perfusion and collateral formation was assessed in the proximal regions of the IL and normalized to the nonischemic limb (NIL). All measurements were performed at 5 days postsurgery. Ischemic limbs were compared between treatment groups. A, Representative LDPI tracings from wild type (WT) and Tg$^{SMC-Cat}$ animals at day 5. B, Quantitative analysis of WT and Tg$^{SMC-Cat}$ LDPI tracings (*P<0.05, n=5). C, Representative micro-CT angiographs from WT and Tg$^{SMC-Cat}$ mice at postoperative day 5. To determine the relative contribution of OPN to collateral formation compared with other H$_2$O$_2$-dependent pathways, we performed an OPN add-back experiment. Tg$^{SMC-Cat}$ lentivirus (LV)-green fluorescent protein (GFP) treatment group had less reperfusion than WT+LV-GFP mice. Add-back of OPN to Tg$^{SMC-Cat}$ mice using LV-OPN (Tg$^{SMC-Cat}$+LV-OPN) restored collateral formation and perfusion to the level of WT animals. D, Representative LDPI tracings from WT+LV-GFP, Tg$^{SMC-Cat}$+LV-GFP, and Tg$^{SMC-Cat}$+LV-OPN treated animals. E, Quantitative analysis of WT+LV-GFP control, Tg$^{SMC-Cat}$+LV-GFP, and Tg$^{SMC-Cat}$+LV-OPN LDPI tracings. (*P<0.001 vs Tg$^{SMC-Cat}$+LV-GFP, n=6). Bars are means±SEM.
were significant decreases in ischemia-induced OPN mRNA and protein expression (Figure 3). In addition, when \( \text{H}_2\text{O}_2 \) levels were decreased using SMC-catalase overexpressing mice, there was a similar decrease in OPN mRNA and protein expression in response to ischemia (Figure 4). Several recent publications link ROS to OPN expression in different in vitro cell systems, including renal epithelial and vascular SMCs. \(^{24-26} \) Consistent with this, our in vitro data also provide evidence that \( \text{H}_2\text{O}_2 \) can directly induce increased OPN expression (Figure 6). The precise mechanism by which \( \text{H}_2\text{O}_2 \) increases OPN in vascular SMCs requires further investigation.

Altogether, these data strongly support a direct relationship between \( \text{H}_2\text{O}_2 \) and OPN expression in vivo, specifically that \( \text{H}_2\text{O}_2 \) produced in response to ischemia increases OPN expression and that SMCs are 1 of the contributing sources of \( \text{H}_2\text{O}_2 \) in this model. As evidenced by immunofluorescence (Figure 1 D–F), SMCs are just 1 cell type that contributes to increased OPN production in the IL. The dramatic effects observed in TgSMC-Cat animals may be due, in part, to SMC-catalase overexpressing cells serving as a sink for \( \text{H}_2\text{O}_2 \), generated by other cell types in response to ischemia because \( \text{H}_2\text{O}_2 \) is freely fusible. In both scenarios where \( \text{H}_2\text{O}_2 \)-dependent OPN expression is decreased, there is substantially impaired reperfusion and collateral formation in response to ischemia compared with controls, where OPN expression is maintained and collateral formation is preserved. Furthermore, when OPN was overexpressed in the ischemic limb of TgSMC-Cat animals using LV, collateral formation and reperfusion were restored to levels similar to those seen in WT+LV-GFP control animals (Figure 5D–E), further substantiating OPN as a critical mediator of postischemic neovascularization.

The experiments performed in this study establish that \( \text{H}_2\text{O}_2 \)-dependent OPN is an important mediator of postischemic neovascularization. One mechanism by which \( \text{H}_2\text{O}_2 \) stimulates collateral vessel formation is through the \( \text{H}_2\text{O}_2 \)-dependent upregulation of OPN expression, which functions to promote neovascularization. Future studies will focus on determining how \( \text{H}_2\text{O}_2 \) regulates OPN expression and defining the pathways activated by \( \text{H}_2\text{O}_2 \) and upstream of OPN. Fully understanding how OPN functions to increase neovascularization may ultimately allow for the development of new therapeutics to increase collateral formation in ischemic tissues to restore blood flow and preserve tissue function.

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Disclosures

None.
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Supplemental Material

Reactive Oxygen Species Regulate Osteopontin Expression in a Murine Model of Post-Ischemic Neo-vascularization

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Extended Materials and Methods

Animals

Male C57BL/6 mice were purchased from Jackson Laboratories (Maine, USA). Transgenic Catalase overexpressing (Tg\textsuperscript{SMC-Cat}) mice were bred in house in the Department of Animal Resources at Emory University. Tg\textsuperscript{SMC-Cat} mice have increased expression of human catalase in vascular tissue through the SMC specific smooth muscle myosin heavy chain (MHC) promoter and were characterized previously.¹ These transgenic mice are on a C57Bl/6 background and for all experiments Tg\textsuperscript{SMC-Cat} mice were compared to wild-type littermate controls. In some experiments, PEG-Catalase dissolved in sterile saline was administered intravenously by osmotic mini-pump at a rate of 10,000 U/kg/day. This concentration was previously reported in the literature, though administered by daily intravenous injection, and was determined to be the dose at which \( \text{H}_2\text{O}_2 \) was significantly decreased in the adductor muscle in this HLI model. Administration of PEG-Catalase began at the time of hind limb surgery and continued for 5 days post-surgery, unless otherwise noted. All animals used were male and between 8 and 10 weeks of age. The animals were housed and cared for according to the guidelines approved by the Emory University Institutional Animal Care and Use Committee.
**Osmotic Mini-pump Implantation**

Mice were pre-anesthetized using 3% isoflurane (oxygen delivered at 0.5L/min with 3% isoflurane for induction and 2.0% isoflurane for maintenance). For PEG-Catalase pumps, fur was removed using clippers and an incision was made in the neck to expose the jugular vein. A jugular catheter attached to a primed osmotic-mini pump (Alzet osmotic mini-pump, model 1007D, Durect Corporation, Cupertino, CA) was inserted and tied into the jugular vein and the pump inserted subcutaneously at the back of the neck. The neck wound was sutured closed and mice were administered Buprenex (0.01-0.1mg/kg, SQ) as needed.

**Hind Limb Ischemia Surgery**

Male mice between 8 and 10 weeks of age were pre-anesthetized with 3% isoflurane in a chamber and then anesthetized with 2% isoflurane through a nose cone. All hair was removed from the surgical site, the area was cleaned with sterile saline, and disinfected with Betadine. Aseptic technique was employed. A unilateral incision was made over the right medial thigh of the mouse. The superficial femoral artery and vein were ligated with 6-0 silk suture proximal to the deep femoral artery branch point, and then a second ligation was performed just proximal to the branching of the tibial arteries. The length of the artery and vein were excised between the two ligation points. The skin was closed with 6-0 silk suture. The animals received Buprenex (0.01-0.1mg/kg, SQ) post-operatively for analgesia as needed. The mice were then allowed to recover on a heated platform.

**LASER Doppler Perfusion Imaging**

LASER Doppler perfusion imaging (LDPI) was completed at 5 days after surgery for each genotype and treatment condition. Mice were anesthetized using inhalation of isoflurane and scanned with the LDPI system (PIM II Laser Doppler Perfusion Imager). Perfusion of the proximal region of the ischemic limb, just below the proximal ligation point, and non-ischemic limb was assessed. Perfusion of the ischemic limb proximal region was quantified and normalized to the same region of the non-surgical limb.
**Micro-CT Imaging**

Quantitative micro-CT was used for evaluation of collateral vessel formation in the ischemic limb at postoperative day 5 as described previously.\(^2,^3\) Briefly, mice were euthanized (n=6-9 for each group) and sequentially perfused with saline containing 4 mg/mL of papaverine, 10% formalin, followed by a lead chromate-based contrast agent (Flow Tech, Inc, South Windsor, CT). Bone was demineralized in formic acid based solution (Cal-Ex II, Fisher Scientific, Pittsburgh, PA) for 48 hours. Samples were then imaged at a 30-μm voxel size, and the tomograms were used to render binarized 3-D images. Stereological algorithms were used to quantify vascular volume to tissue volume ratio, connectivity, and vascular density, which were then normalized to the contralateral control limb.

**Lentivirus**

The viral vector was derived from the HIV-based lentivirus backbone pLV-CMV-GFP-U3Nhe, as described previously,\(^4,^6\) and was a kind gift of Dr. Kerry J. Ressler. The pLV-CMV-GFP-U3Nhe vector allows for virally mediated expression of green fluorescent protein (GFP) driven by a cytomegalovirus (CMV) promoter. pLV-CMV-GFP-U3Nhe control vector will hereafter be referred to as LV-GFP. We then generated a dual-tagged human Osteopontin (OPN) expressing an HA-tag at the N-terminus and a Myc-tag at the C-terminus. We then inserted a wild-type internal ribosome entry site (IRES),\(^7\) followed by the dual-tagged full-length OPN downstream of GFP to generate a lentivirus that expresses both GFP and tagged OPN, hereafter referred to as LV-OPN. The wild-type IRES used has been shown previously to promote high translation of the downstream, compared to the upstream coding sequence in the same mRNA,\(^7\) thereby allowing the final construct to produce high OPN and relatively low GFP protein.

Viral production procedures have been described in detail previously.\(^4,^8,^9\) In brief, active viral particles were produced by co-transfecting these lentiviral packaging constructs with
plasmids coding for delta8.9 and VSV-G into HEK-293T cells. The packaged, unconcentrated virus was collected over a period of 5 days post-transfection, and then concentrated using ultracentrifugation and resuspension in sterile PBS/1% BSA. The resulting titer was assessed in HEK-293T cells, and the observed titer of the LV-GFP and LV-OPN expressing viruses used here were each ~2x10⁹ infectious particles per mL. For in vivo use, both lentiviruses were diluted to a final concentration of ~1x10⁹ infectious particles per mL. All animals received HLI. After the artery and vein were tied off and excised, the adductor muscle (inner thigh) was injected with 20 μL of either LV-GFP lentivirus or LV-OPN lentivirus using a similar approach as described previously.⁴

Detection of ROS

To evaluate intracellular production of O₂⁻, we used DHE-HPLC to measure the conversion of 2-hydroxyethidium from DHE using high-performance liquid chromatography (HPLC). Hydrogen peroxide measurements in intact tissues were made using the Amplex Red Assay in which we measured the oxidation of Amplex Red (100 μmol/L, Sigma-Aldrich, St. Louis, MO) in the presence of horseradish peroxidase using the Amplex Red Assay Kit (Molecular Probes, Carlsbad, CA), as described previously (Dikalov et al., 2008). Briefly, Amplex Red and horseradish peroxidase type II (0.1 U/mL) in Krebs HEPES buffer were added to the tissue samples. Fluorescence readings were made in triplicate in a 96-well plate at Ex/Em=530/580 nm using 100-μL of Amplex/Krebs buffer in which the tissue was incubated. H₂O₂ production was calculated and normalized to tissue wet weight.

Immunofluorescence

Mice were sacrificed at indicated times and tissues were perfused in situ with saline and then fixed with 10% buffered formalin. Tissue sections from paraffin embedded proximal hindlimbs were paraffin embedded and cut into 5 μm sections. Antigen retrieval was performed in citrate buffer, pH 6.0 (Invitrogen, Carlsbad, CA), prior to incubating with primary antibodies.
Osteopontin antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) and R&D Systems (Minneapolis, MN) were used at a dilution of 1:200 in 3% BSA, followed by incubation with Horse anti-Mouse secondary antibody (Vector Labs, Burlingame, CA) or Donkey anti-Goat secondary antibody at a dilution of 1:400 in 3% BSA, and finally incubated in Streptavidin QDot 655 (Invitrogen, Carlsbad, CA) at a dilution of 1:200 in 3% BSA. For double staining experiments, Mac3 antibody (BD Pharmingen, San Diego, CA) was used at a dilution of 1:50, Lectin antibody (Vector Labs, Burlingame, CA) was used at 1:100, and smooth muscle alpha-actin antibody (Abcam, Cambridge, MA) was used at 1:200. Images of the sections were collected using the 20X and 40X Plan-Neo air objectives (Numerical aperture 0.50) on a Zeiss Axioskop Microscope equipped with an AxioCam camera.

**Immunoblotting**

Adductor muscle tissue were homogenized with glass mortar and pestle or VSMCs were homogenized in Hunter’s Lysis Buffer (25 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EGTA, 10 mmol/L Na-pyrophosphate, 10 mmol/L NaF, 0.1 mmol/L Na-orthovanadate, 1% Na deoxycholate, 1% Triton X 100, 0.1% SDS, 10% Glycerol, and protease inhibitors) for all experiments. Homogenates were rocked with end-over-end rotation at 4°C for 20 minutes to allow for complete tissue or cell lysis. Cell samples were sonicated on ice at 10 watts for 10 x 1 second pulses to further disrupt the cell membrane using a Microson Ultrasonic Cell Disruptor XL (Misonix, Inc., Farmingdale, NY). Whole cell or whole tissue lysates were utilized for Western Blot (WB) experiments. Protein concentrations were assessed using the Bradford assay (BioRad, Hercules, CA). For WB analysis, proteins were separated using SDS-PAGE and transferred to nitrocellulose membranes, blocked, and incubated with the appropriate primary antibodies. The Osteopontin antibodies used were from Santa Cruz Biotechnology (Santa Cruz, CA) and R&D Systems (Minneapolis, MN). GAPDH antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The β-Actin antibody was from Cell Signaling (Danvers, MA). HRP-conjugated secondary antibodies (BioRad, Hercules, CA) were used for
detection using enhanced chemiluminescence (ECL; GE Healthcare UK, Little Chalfont, Buckinghamshire, UK). Band intensity was quantified by densitometry using ImageJ 1.38 software.

**Cell Culture**

Vascular smooth muscle cells from mouse aorta (MASMs) were isolated (passages 4-10) and cultured in Dulbecco’s Modified Eagle’s Media (DMEM; Sigma Aldrich, Saint Louis, MO) supplemented with 10% Fetal Bovine Serum (FBS; Sigma Aldrich, Saint Louis, MO), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin, all of which were obtained from Mediatech, Inc. (Manassas, VA). Cells were stimulated with H$_2$O$_2$ after 48 hours of quiescence in serum free DMEM for all experiments.

**RNA Isolation and Quantitative RT-PCR**

Total RNA was extracted from muscle tissue or VSMCs using the RNeasy kit (Qiagen). Superscript II (Invitrogen) and random primers were used for RT. OPN (primer sequences: GTATGAGACGGGACAGCTATTTCTCCA and CTGACATAGTCCAAGCCTGGGATG) and 18S rRNA, were measured by amplification of cDNA from muscle tissue or VSMCs using the LightCycler real-time thermocycler and SYBR green dye. All are normalized to 18S rRNA.

**Statistical Analysis**

Results are expressed as mean ± S.E.M. from at least three independent experiments. Statistical significance for quantitative results was assessed using analysis of variance (ANOVA), followed by Bonferroni’s Multiple Comparison post-hoc test. In some cases, a t-test was used to assess significance. A value of p<0.05 was considered statistically significant.
Supplemental References

7. Bochkov YA, Palmenberg AC. Translational efficiency of emcv ires in bicistronic vectors is dependent upon ires sequence and gene location. *Biotechniques*. 2006;41:283-284, 286, 288 passim
Supplemental Figure Legends

Supplemental Figure I. Expression of Inflammatory Markers in the Proximal Region of the Ischemic Limb is H$_2$O$_2$-dependent. mRNA levels of the inflammatory markers MCP-1 and TNF-α were measured in the adductor muscles of the non-ischemic (NIL) and ischemic limbs (IL) at 5 days after femoral artery ligation in Tg$^{SMC-Cat}$ mice or wild-type littermate controls (WT) and in C57Bl/6 mice infused with Saline or 10,000 U/kg/day PEG-Catalase (PEG-Cat).

A. Ischemia significantly increased MCP-1 in the IL of WT animals (p<0.05 vs. WT NIL), which was blunted in the IL Tg$^{SMC-Cat}$ mice (p<0.02 vs. WT IL).

B. TNF-α increased in the IL of WT animals (p<0.01 vs. WT NIL), which was blunted in the IL Tg$^{SMC-Cat}$ mice (p<0.0001 vs. WT IL).

C. MCP-1 expression was increased in the IL of Saline control animals (p<0.001 vs. Saline NIL), which was blunted in the IL of PEG-Cat infused (p<0.0001 vs. Saline IL).

D. TNF-α increased in the IL of WT animals (p<0.01 vs. WT NIL), which was blunted in the IL Tg$^{SMC-Cat}$ mice (p<0.0001 vs. WT IL). Data are expressed as fold change compared with WT NIL or Saline NIL. Bars are means ± S.E.M., n=5-6 per genotype/treatment.

Supplemental Figure II. H$_2$O$_2$-dependent OPN Mediates Collateral Formation. To determine the functional importance of PEG-Catalase mediated decreases in H$_2$O$_2$ and OPN on collateral formation, we used LASER Doppler Perfusion Imaging (LDPI) to evaluate reperfusion and Micro-CT to quantify collateral formation in the IL. Perfusion and collateral formation was assessed in the proximal regions of the IL and were normalized to the NIL. All measurements were performed at 5d post-surgery. IL were compared between treatment groups.

A. Representative LDPI tracings from Saline and PEG-Catalase infused animals.

B. Quantitative analysis of Saline and PEG-Catalase LDPI tracings (*p<0.01, n=5).

C. Representative Micro-CT angiographs from Saline and PEG-Catalase mice.

Supplemental Figure III. Lentivirus Delivery of Myc-tagged OPN or GFP in the Ischemic Leg. For in vivo use, both the LV-GFP and LV-OPN lentiviruses were diluted to a final
concentration of $\sim 1 \times 10^9$ infectious particles per mL. All animals received HLI. After the artery and vein were tied off and excised, the adductor muscle was then injected with 20 μL of either LV-GFP or LV-OPN lentivirus. To verify that cells within the muscle were successfully transduced by lentivirus, we performed immunofluorescence staining. A. WT animals transduced with LV-GFP were positive for GFP (red) and negative for Myc-tagged OPN (green). B. We also show that Tg$^{SMC-Cat}$ adductor muscles were successfully transduced by LV-OPN lentivirus, as shown by Myc staining for Myc-tagged OPN (green) in LV-OPN animals, whereas Tg$^{SMC-Cat}$+LV-GFP show no Myc expression. Because TgSMC-Cat animals express GFP in all tissues, due to the nature of the transgene, these sections were only stained with Myc to detect successful expression of Myc-OPN and were not co-stained for GFP.
Supplemental Figure II

A

Saline

PEG-Catalase

Perfusion Ratio (IL:NIL)

B

Saline PEG-Cat

C

Saline

PEG-Catalase

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Supplemental Figure III

A  WT + LV-GFP

B  Tg^{SMC-Cat} + LV-GFP  Tg^{SMC-Cat} + LV-OPN