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Control of Angiogenesis Dictated by Picomolar Superoxide Levels

Shyamal C Bir¹, Xingguo Shen¹, Terrance J Kavanagh³, Christopher G Kevil^{1,2}, and Christopher B Pattillo^{2,*}

¹Department of Pathology, LSU Health Sciences Center-Shreveport, Shreveport, LA 71104

²Department of Molecular and Cellular Physiology, LSU Health Sciences Center-Shreveport, Shreveport, LA 71104

³Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA 98195

Abstract

Control of vascular insufficiencies due to various cardiovascular pathologies is important for developing specific and effective treatments. Fluctuations in oxidative stress significantly alter the progression of angiogenesis under physiological and pathological conditions. However, the precise amount of reactive oxygen species (ROS) required to influence subsequent signaling pathways for ischemic angiogenesis remain undefined. Here, we have determined the effect of ROS mediated molecular mechanisms on angiogenesis in a murine model of peripheral artery disease using Gclm mutant mice (a model of compromised glutathione synthesis, therefore reduced antioxidant capacity). Left femoral artery ligation and excision were performed in Gclm WT (+/+), heterozygous (+/-), and null (-/-) mice. Blood flow (laser Doppler), angiogenic index (CD31/DAPI) and proliferation index (Ki67/DAPI) were significantly increased in Gclm^{+/-} mice but not in Gclm^{+/+} or Gclm^{-/-} mice. Measurements of reactive oxygen species suggest that the amount of superoxide required to stimulate angiogenesis following the induction of ischemia is 9.82 pmol/mg of tissue. Protein carbonyl levels increased in a manner consistent with increasing oxidative stress. Superoxide and protein carbonyl levels were reduced by the addition of the nitroxide tempol, a known superoxide dismutase mimetic. Finally, restoration of blood flow in Gclm^{+/-} mice was attenuated by a VEGF₁₆₄ aptamer verifying that slightly elevated levels of ROS restore blood flow by stimulating endothelial cell proliferation through a VEGF dependent pathway. The results of this study reveal new information on the amount of ROS necessary for angiogenic activity and provide the foundation of critical redox parameters for vascular remodeling responses. The information obtained from this study on vascular ischemia, using a model of decreased antioxidant capacity, has provided insight into the control of revascularization and is a step forward in our ability to regulate angiogenic therapies.

Keywords

glutathione; Gclm; oxidative stress; angiogenesis; PAD

*Correspondence to: Christopher B. Pattillo, Ph.D., Department of Molecular and Cellular Physiology, LSU Health Sciences Center-Shreveport, 1501 Kings Hwy, Shreveport, LA 71130, cpatt7@lsuhsc.edu, Phone: (318) 675-6974, Fax: (318) 675-6005.

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Introduction

Vascular growth and remodeling processes following ischemia are extremely important in restoring oxygen homeostasis. This is accomplished through various mechanisms of vascular development, which involve angiogenesis (increased capillary growth), arteriogenesis (collateral artery remodeling and growth), and vasculogenesis (progenitor cell influenced growth) [1]. Previous work has demonstrated that vascular growth and remodeling are regulated by various cytokines (VEGF, bFGF, PDGF-BB, SDF-1, etc.), and signaling mediators (Akt, ERK 1/2, and others), which drive other cellular responses including activation, proliferation, migration and maturation of endothelial cells [1, 2]. However, all of these mediators and signaling pathways are also critically influenced by redox biology, and the role reactive oxygen species (ROS) play in these processes is still poorly defined. Previous findings suggested that ROS stimulates an angiogenic response in hearts during ischemia reperfusion [3]. A recent study of restenosis following a stent graft demonstrated that oxidative stress causes vessel wall thickening by extensive neovascularization [1]. Angiogenesis following femoral artery ligation is mediated by VEGF induced reactive oxygen species production [4]. These findings suggest that ROS induces tissue injury in some cases, but is also capable of promoting tissue regeneration in others. In the present study, we have determined the amount of ROS required to stimulate angiogenesis and the molecular mechanism related to ROS induced ischemic vascular growth through the use of Gclm mutant mice with compromised glutathione synthesis.

Glutathione (GSH) is found in all mammalian tissues, and represents the most abundant cellular low molecular weight thiol [5]. Glutathione is present in reduced (GSH) and oxidized (GSSG) forms [6], and regulates the intracellular redox state by scavenging radicals and products of lipid peroxidation [7]. GSH is produced inside cells in two ATP-dependent reactions catalyzed by glutamatercysteine ligase (GCL) and GSH synthetase [6–8]. GCL, a heterodimeric enzyme consisting of catalytic (GCLC) and modifier (GCLM) subunits, regulates the ratelimiting step in GSH synthesis. GCLM augments the catalytic activity of GCLC by decreasing the K_m for glutamate and ATP, and increasing the K_i in the case of GSH feedback inhibition [7–9]. Both GCLC and GCLM genes have oxidative stress-responsive elements in their promoter-enhancer regions [9–11] and transcription is induced following oxidative insult. Previous reports show that deletion of the Gclc gene in mice results in an embryonic lethal phenotype [12] [13], whereas Gclm deletion results in viable offspring. Although Gclm null mice are in apparent good health, they have a chronic reduction of GSH in the mitochondria and tissues [14] that has been linked to altered vascular reactivity possibly due to enhanced production of nitrotyrosine [15], and are sensitive to cardiac ischemia reperfusion injury, and will develop dilated cardiomyopathy in a pressure overload model [3, 16]. Thus, Gclm heterozygous and null mice are considered a useful animal model for understanding the role of GSH and the factors that control its synthesis in several pathological conditions including ischemic vascular remodeling where oxidative stress is a real concern [10, 17].

Materials and Methods

Reagents

Anti-CD31 and anti-Ki67 antibodies were obtained from BD Biosciences. Vectashield plus DAPI was obtained from Vector Laboratories. Cy3 conjugated secondary antibodies were purchased from Jackson ImmunoResearch Inc. Dihydroethidium and Tempol were purchased from Sigma and Enzo Life Sciences respectively.

Animal model

Gclm^(-/- or +/-) mutant mice were bred and housed at the Association for Assessment and Accreditation of Laboratory Animal Care, internationally accredited Louisiana State University Health Science Center-Shreveport animal resource facility, and maintained in accordance with the National Research Council's Guide for Care and Use of Laboratory Animals. All animal studies were approved by the institutional animal care and use committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

Hind limb ligation

Chronic hind limb ischemia was induced in Gclm^(-/- or +/-) mice by ligating and transecting the left common femoral artery as well as its collateral branches as we have previously reported [18, 19]. Briefly, mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (8 mg/kg). After measuring hind limb blood flows using laser tissue Doppler, aseptic surgery was performed by a linear incision at the left groin, exposing the left common femoral artery identified by its pale pink color and pulsatile nature. Immediate pallor was observed in the distal hind limb following ligation of the artery using 5-0 silk. Laser Doppler was taken pre-, post-, and days 1, 3, 5, 7 following surgical induction of hind limb ischemia.

Laser Doppler tissue blood flow

The Vasamedics Laserflo BPM2 deep tissue laser Doppler device was utilized to measure hind limb blood perfusion as we previously reported [18, 19]. The laser probe was placed over the medial gastrocnemius muscle region of both the hind limbs of the mice. Readings were recorded in milliliter of blood flow per 100 g tissue per minute. Percent blood flows

were determined as: $\left[\frac{a}{b} \times 100 \right]$ where a = ischemic limb average flow and b = non-ischemic limb average flow.

Vascular density measurement

Immunohistochemistry was performed as we have previously described with anti- CD31 antibody to examine the progression of proliferation and angiogenesis [18, 19]. Gastrocnemius muscles from ischemic and non-ischemic hind limbs were collected and embedded in OCT freezing medium, frozen, and cut into 5 μ m sections. Specimens were fixed at -20°C in 95% ethanol/5% glacial acetic acid for 1 h, washed three times in cold PBS with 1% horse serum (5 min per wash) and blocked overnight with 5% horse serum in PBS at 4°C. Anti-CD31 (platelet/endothelial cell adhesion molecule-1 [PECAM-1]) was added at 1:200 dilution (in PBS with 0.05% horse serum) and incubated at 37°C for 1 h. Slides were then washed and Cy3 conjugated secondary antibody was added at 1:250 dilution (in PBS with 0.05% horse serum) and incubated at room temperature for 1 h. Slides were rinsed with PBS and 0.05% horse serum and mounted using Vectashield DAPI (4',6-Diamidino-2'-phenylindole dihydrochloride) medium. Four slides per hind limb with three sections per slide were used for vascular density analysis. Images were taken with a Hamamatsu digital camera using a Nikon TE-2000 epifluorescence microscope (Nikon Corporation, Japan) at 200x magnification. Simple PCI software version 6.0 (Compix Inc., Sewickly, PA, USA) was used to measure the area of CD31 staining.

Cellular proliferation measurement

Immunofluorescent staining of the nuclear cell proliferation protein Ki67 was utilized to detect proliferating cells (anti-Ki67 1:350 dilution) from total cells (DAPI staining) per section, as we have previously described [18]. Frozen tissue sections were stained for Ki67,

CD31, and DAPI to identify colocalized markers indicating endothelial cell proliferation. Images were acquired as outlined above. Cellular proliferation was determined as the ratio between regions positive for Ki67 and DAPI positive areas.

Tissue superoxide measurements

Tissue superoxide production was measured using the hydroethidine (HE) HPLC method as previously reported [20]. On day 3 and 7, all mice were injected IP with 300 μ l of dihydroethidium (1 μ g/ μ l). Dihydroethidium injected animals were sacrificed one hour later and gastrocnemius muscle tissue from non-ischemic and ischemic hind limbs were isolated and cut in a mid-sagittal manner resulting in two equal portions of gastrocnemius tissue. Tissue was homogenized in a 50 mM phosphate buffer (pH 7.4) with 5 mM potassium cyanide (KCN), divided into two fractions and one half was precipitated using acidified methanol; 2-OH-E⁺ was enriched from the supernatant of the second fraction after precipitating protein, using a micro-column preparation of Dowex 50WX-8 cation exchange resin and eluted with 10 N HCl. 2-OH-E⁺ product was then measured using fluorescence detection (ex: 490; em: 567) with a Shimadzu UFLC HPLC system. 2-OH-E⁺ superoxide production was normalized to total protein and reported as pmol/mg of protein.

Tissue GSH / GSSG measurements

GSH/GSSG was measured as we have previously described [21]. Briefly, ischemic tissue from gastrocnemius muscle was collected from Gclm^(+/-) and ^(-/-) mice. Tissues were homogenized in PBS (pH 7.4) containing 50 mM Nethylmaleimide, proteins precipitated with 5% TCA and samples centrifuged at 12,000 $\times$ g for 8 min. Excess N-ethylmaleimide was extracted from the supernatants with 10 volumes of dichloromethane and samples were centrifuged again. After alkalization of the upper phase (0.1 ml sample + 0.05 ml Tris-HCl 1M, pH 10.0, adjust pH to 7.5~8.0), samples were derivatized with 1-fluoro-2, 4-dinitrobenzene (DNFB) for 2 hours, protected from light, at room temperature. After adding 10 μ l HCl (37% v/v), samples were analyzed by Shimadzu Prominence Ultra Fast Liquid Chromatography using a LiChrosorb NH₂ column (250 $\times$ 4.6 mm), a UV detector (355 nm) and a gradient elution (mobile phase: 80% methanol and acetate buffer 0.5M, pH 4.6, 64% methanol).

Tissue protein carbonyl measurements

Tissue protein carbonyl concentrations were measured from the ischemic gastrocnemius muscle using a Protein Carbonyl ELISA from Cell Biolabs Inc (San Diego, CA) according to the manufacturer's instructions [20]. Protein carbonyl formation was normalized to total protein and reported as nmols/mg of protein.

VEGF measurement

Ischemic and non-ischemic tissues from gastrocnemius muscle were collected from Gclm^(+/-) and ^(-/-) mice. Vascular endothelial growth factor was measured using an ELISA kit (R&D), and following manufacturer's instructions [22].

Statistical analyses

Two-way Analysis of Variance with Newman-Keuls post hoc test were used to statistically compare changes in ischemic hind limb blood flow with laser Doppler to compare effects of time as well as treatment. A p-value of <0.05 was required for statistical significance. Statistics were performed with GraphPad Prism 4.0 software. The number of mice used per experiment is reported in the figure legends.

Results

Decreased superoxide increases blood flow by stimulating proliferation and vascular density in hind limb ischemia

There is evidence to suggest that physiological doses of reactive oxygen species are safe and beneficial for restoration of blood flow [23]. Therefore, we have investigated the effect of ROS on angiogenesis in Gclm^{+/-} and ^{-/-} mice, which have been shown to have compromised GSH synthesis. Figure 1A shows that blood flow in Gclm^{+/-} mice was significantly increased by day 3 post ligation compared to WT and Gclm^{-/-} mice and this increased flow was maintained until the end of the study period. The elevation in blood flow was attributed to elevated ROS, one in particular, superoxide, is a predominant effector of cell signaling. To determine what role superoxide plays in the revascularization process we employed the use of tempol, a nitroxide and superoxide dismutase (SOD) mimetic which metabolizes superoxide to hydrogen peroxide through catalytic action [24]. Tempol significantly attenuated the ability of superoxide to restore blood flow in Gclm^{+/-} mice following hind limb ischemia (figure 1B). Successful restoration of blood flow in ischemic tissue corresponded to enhanced vascular density. Figure 1C shows that vascular density (the ratio of CD31 [endothelial stain] and DAPI [nuclear counter stain]) was significantly increased at day 7 in the ischemic muscle tissue of Gclm^{+/-} mice compared to WT or Gclm^{-/-} mice. Both blood flow and angiogenic activity were blunted by tempol in the ischemic hind limbs of Gclm^{+/-} mice 7 days post ligation. One requirement for angiogenic activity is the proliferation of endothelial cells [25]. Figure 1D shows that endothelial cell proliferation (ratio of Ki67 [proliferation marker] staining and DAPI) was significantly increased at day 7 in the ischemic muscle tissue of Gclm^{+/-} mice compared to WT or Gclm^{-/-} mice, but tempol inhibited this response. These data indicate that superoxide is highly effective in the restoration of blood flow in the ischemic tissue of Gclm^{+/-} mice by increasing proliferation and angiogenic activity of the endothelium.

Redox activity in ischemic tissue of Gclm mutant mice 3 days post hind limb ligation

Currently, there is no evidence describing levels of superoxide or GSH necessary to stimulate angiogenesis in ischemic tissue. The ratio of reduced to oxidized glutathione is very important in regulation of oxidative stress, particularly during ischemic conditions. Levels of GSH in Gclm^{+/-} mice were measured and found to fall between those of WT and Gclm^{-/-} mice (figures 2A) and although the GSH/GSSG ratio appeared to be increased in Gclm^{+/-} mice this did not achieve statistical significance (figure 2B). Levels of superoxide were then measured in ischemic muscle tissues of Gclm mutant mice. Figure 2C shows that there was no significant difference in plasma superoxide levels irrespective of Gclm genotype and that superoxide levels in the ischemic tissue of Gclm^{+/-} mice were between that of WT and Gclm^{-/-} mice (figure 2D). The approximate amount of superoxide required for the restoration of blood flow in Gclm^{+/-} mice, was 9.82 pmol/mg of tissue, 3 days post ligation. These data suggest that approximately 9.82 pmol/mg of superoxide is conducive to angiogenesis whereas levels higher (13.97 pmol/mg for Gclm^{-/-}) or lower (7.63 pmol/mg for WT) provide an environment detrimental to proliferation, angiogenesis and blood flow restoration in the ischemic hind limb of mice. Importantly tempol reduced the superoxide levels in ischemic tissues of Gclm^{+/-} mice and abolished both the increased blood flow and angiogenesis observed in these animals (figures 2D, 1B, and 1C respectively).

Redox activity in ischemic tissue of Gclm mutant mice 7 days post hind limb ligation

We have also measured the GSH, GSH/GSSG and superoxide level in ischemic muscle tissue of Gclm mutant mice at day 7. The GSH level in the Gclm^{+/-} mice was in between that of WT and Gclm^{-/-} mice (figure 3A). GSH/GSSG ratio was significantly increased in ischemic tissue of Gclm^{+/-} mice compared to those of WT and Gclm^{-/-} mice (figure 3B).

There was no difference in plasma superoxide levels found in these three groups of mice (figure 3C). The superoxide level in ischemic tissue in Gclm^{+/-} mice (11.38 pmol/mg) was in between WT (7.01 pmol/mg) and Gclm^{-/-} mice (15.16 pmol/mg). The use of tempol reduces the superoxide levels in the ischemic tissues of Gclm^{+/-} mice (figure 3D) similar to day 3 post ligation.

Protein carbonyl levels in ischemic tissue of Gclm mutant mice 7 days post ligation

Carbonylation of protein is highly related to oxidative stress [20]. Excessive carbonylation of protein has been associated with various pathological states [20]. Therefore, we have also examined the protein carbonyl levels in ischemic muscle tissue of Gclm mutant mice at day 7. Figure 4 shows that the protein carbonyl level in Gclm^{+/-} mice was in between that observed for WT and Gclm^{-/-} mice. The addition of tempol reduced protein carbonyl levels back to WT levels in Gclm mutant mice (although levels were still significantly higher than WT not treated with tempol). These data combined with previous figures indicate that oxidative stress within a narrow range can stimulate angiogenesis and restore blood flow in Gclm^{+/-} mice following induction of hind limb ischemia.

Superoxide restores blood flow in Gclm^{+/-} mice by a VEGF dependent pathway

Superoxide can activate different angiogenic signaling pathways, one of which is the VEGF signaling pathway [26]. We explored the role VEGF plays in superoxide-induced angiogenesis in Gclm mutant mice following hind limb ligation. Figure 5A shows that VEGF is increased in the ischemic tissue of Gclm^{+/-} mutant mice compared to WT and Gclm^{-/-} mice; the addition of tempol attenuates the increase in VEGF levels. Figure 5B illustrates that the restored blood flow in Gclm^{+/-} mice was blunted by an aptamer that is a competitive antagonist to VEGF₁₆₄; a denatured form of the aptamer (DN-VEGF Aptamer) was used as a negative control. Consistent with the blood flow data, figures 5C and D indicate that the angiogenic and proliferative activity were also attenuated by the addition of the VEGF₁₆₄ aptamer. Finally, the use of the VEGF aptamer attenuated the VEGF level in the ischemic muscle tissue of Gclm^{+/-} mice (figure 5E). Restoration of blood flow in Gclm^{+/-} mice was also blunted by the VEGF₁₆₄ aptamer indicating VEGF₁₆₄ as an important ROS dependent ischemic angiogenesis mediator.

Discussion

The major findings of this study are that picomolar levels of superoxide are sufficient to cause major changes in the restoration of blood flow in Gclm mutant mice, an effect that is blunted by treatment with tempol. We also demonstrated that superoxide stimulates vascular growth in the ischemic tissue of Gclm^{+/-} mice through a VEGF dependent pathway. Figure 6 is a summary of our findings for this manuscript. Three genetic mutants were used a Gclm WT (+/+), Gclm heterozygote (+/-), and a Gclm knock out (-/-). Following hindlimb ligation the three groups of animals had levels of tissue superoxide which correlated to reductions in glutathione synthesis. Animals with severely diminished glutathione levels (Gclm^{-/-}) had the highest levels of tissue superoxide and the animals with normal glutathione levels (Gclm^{+/+}) had the least amount of measureable tissue superoxide levels. The heterozygous Gclm mutant produced levels of superoxide that were between those of the homozygote and wild type animals, these levels provided the ideal stimulus which lead to VEGF dependent angiogenesis.

Generally antioxidants are present that maintain basal levels of ROS and can prevent oxidative injury following ischemia. When the cellular compartment is in an oxidized state or there is abundant oxidized glutathione (GSSG) more ROS are present then in the basal state [7, 8]. Data presented here show that superoxide levels increased and GSH levels

decreased in Gclm^{+/-} mice, while Gclm^{-/-} mice (more severe reduction in basal GSH levels) showed even higher levels of superoxide and a more pronounced decrease in GSH levels following ischemia (figures 2 and 3). ROS demonstrate a biphasic effect in regard to cellular activity; low levels of ROS induce proliferation and growth of vascular cells, however high levels of ROS stimulate apoptosis and necrosis [23]. Literature has shown that ROS induces tubular morphogenesis of endothelial cells *in vitro* [27], and *in vivo* studies have demonstrated that ROS can stimulate intracellular signaling, positively augmenting angiogenesis [23]. The aforementioned evidence shows that ROS can stimulate physiological angiogenesis but ROS has also been linked to pathologies including tumor angiogenesis and angiogenic retinopathy [28, 29]. Currently there are no clear data conveying the required amounts of ROS that influence physiological or pathological angiogenesis. Here we present data that suggest that 9.82 pmol/mg of ROS initiates physiological angiogenesis 3 days post ligation in a model of hind limb ischemia in Gclm^{+/-} mice, an effect maintained until 7 days post ligation. The use of tempol resulted in decreased levels of ROS and protein carbonyl, which correlated with a decrease in blood flow restoration and angiogenesis. These data make a strong case that low levels of ROS promote a robust increase in angiogenic activity in response to ischemia, and these levels are somewhere between those of wild type animals (low) and Gclm knock out animals (high). Various enzymatic and nonenzymatic antioxidants attenuate ROS mediated injury within endothelial cells [30]. When generation of ROS outpaces the supply of antioxidants the cell loses its redox homeostasis and the subsequent injury is associated with several pathologies; lower levels of ROS, however, serve as signaling molecules that mediate endothelial cell proliferation and migration [28, 31].

VEGF is one of the prime pro-angiogenic cytokines that stimulate angiogenesis and vasculogenesis under physiological and pathological conditions [32]. VEGFR-1 (Flt-1) is effective in growing stable new vessels in cardiac and limb ischemia through its actions on endothelial cells, smooth muscle cells, and inflammatory cells [33]. VEGF induces various endothelial cell (EC) responses including EC remodeling through VEGFR-1/Flt-1 and VEGFR-2/Flk-1/KDR that are necessary for vascular development during ischemic angiogenesis [34, 35]. Stimulation of VEGFR-1/Flt-1 has been implicated in EC migration. The action of the VEGFR-2/Flk-1/KDR receptor is associated with both EC migration and proliferation activating numerous downstream signaling molecules including PKC, MAP Kinase/ERK1/2 cascade, c-Src, and PI3 kinase-Akt/protein kinase B, p38 and phospholipase C [32, 36, 37]. Evidence suggests that ROS production through activation of NADPH oxidases, leads to VEGFR2 autophosphorylation, and angiogenesis defined by endothelial cell migration and proliferation [28, 31].

Genetic deficiency of NADPH (NOX1) and the subsequent reduced production of superoxide attenuate angiogenic activity in mice [38]. Importantly, NOX1 knockout mice were found to have reduced matrigel plug and tumor angiogenesis responses along with decreased endothelial cell migration and tube formation *in vitro*. Overexpression of NOX4 augments ROS production that, as a result, stimulates VEGFR2 autophosphorylation and Akt activation, thereby promoting EC migration and proliferation, an effect that is attenuated by using NOX4-siRNA [39, 40]. This study shows that low levels of ROS increase VEGF to stimulate angiogenesis and VEGF antagonists blunt ROS induced ischemic vascular growth and remodeling. These data highlight the important role of VEGF in ROS induced ischemic vascular remodeling.

Taken together, the findings in this study suggest that low levels of superoxide (9.82 – 11.38 pmol/mg) stimulate angiogenesis and restore blood flow in ischemic tissue of Gclm^{+/-} mice via a VEGF dependent pathway. This information may lead to new tailored methodologies for the treatment of patients with peripheral arterial disease. Moreover, it suggests that

although individuals harboring 5' promoter polymorphisms in GCLC or GCLM may be at elevated risk for coronary artery disease, they may at the same time be prone to enhanced angiogenesis and vasculogenesis and accelerated recovery of blood flow to ischemic tissue [41–43]. These polymorphisms would also be predicted to predispose to other pathologies such as atherosclerosis, retinopathies, and solid tumors where increased angiogenesis and vasculogenesis can exacerbate such diseases.

Conclusions

Tissue ischemia following peripheral artery disease is a direct result of blocked or diminished blood flow. The present body of work demonstrates novel data linking glutathione levels and revascularization in a murine model of PAD. Slightly diminished levels of glutathione synthesis capacity have a significant impact in the propagation of angiogenesis. This slight reduction in glutathione allows just enough superoxide production to stimulate a VEGF-dependent robust increase in tissue vasculature. This manuscript identifies a unique paradigm of thresholding ROS levels to produce tailored biological responses, an effect which, following further study, may be used to design effective therapies for ischemic disease.

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- Heterozygous deletion of *Gclm* results in partially reduced glutathione synthesis.
- Reduced glutathione enhances vascularity in a model of peripheral artery disease.
- Picomolar levels of superoxide control VEGF production and vascular remodeling.

Figure 1.

Low levels of ROS stimulate angiogenesis and restore blood flow to ischemic limbs of Gclm^{+/-} mice. Panel A represents blood flow data following induction of hind limb ischemia in Gclm WT, +/- and -/- mice. Panel B shows blood flow data following induction of hind limb ischemia and treatment with tempol (ROS scavenger) in the Gclm^{+/-} mouse. Panel C reports the quantitative measurement of the CD31:DAPI ratio from images of ischemic tissues at day 7 following ligation and treatment with tempol. Panel D shows the quantitative measurement of the Ki67:DAPI ratio from images of ischemic tissues at day 7 following ligation and treatment with tempol. n=5 per cohort, *p<0.05 (compared to WT), #p<0.05 (compared to Gclm^{+/-})

Figure 2.

Glutathione and superoxide levels vary 3 days post ligation in ischemic tissues of Gclm WT, +/- and -/- mice. Panel A represents the glutathione level following induction of hind limb ischemia in Gclm WT, +/- and -/- mice. Panel B reports the reduced and oxidized glutathione ratio (GSH/GSSG) following induction of hind limb ischemia in Gclm WT, +/- and -/- mice. Panel C shows the plasma superoxide level following induction of hind limb ischemia and the levels of superoxide following the addition of tempol in Gclm WT, +/- and -/- mice. Panel D represents the ischemic tissue superoxide level following induction of hind limb ischemia and the levels of superoxide following the addition of tempol in Gclm

WT, +/- and -/- mice. n=3 per cohort, *p<0.05 (compared to WT), #p<0.05 (compared to Gclm^{+/-})

Figure 3.

Glutathione and superoxide levels vary 7 days post ligation in ischemic tissues of Gclm WT, +/- and -/- mice. Panel A represents the glutathione level following induction of hind limb ischemia in Gclm WT, +/- and -/- mice. Panel B reports the reduced and oxidized glutathione ratio (GSH/GSSG) following induction of hind limb ischemia in Gclm WT, +/- and -/- mice. Panel C shows the plasma superoxide level following induction of hind limb ischemia with and without the addition of tempol in Gclm WT, +/- and -/- mice. Panel D represents the ischemic tissue superoxide level following induction of hind limb ischemia with and without the addition of tempol in Gclm WT, +/- and -/- mice. n=3 per cohort, *p<0.05 (compared to WT), #p<0.05 (compared to Gclm^{+/-})

Figure 4. Protein carbonyl levels at day 7 in Gclm WT, +/- and -/- mice. Panel D represents protein carbonyl levels from ischemic tissue following induction of hind limb ischemia with and without the addition of tempol in Gclm WT, +/- and -/- mice. n=3 per cohort, *p<0.05 (compared to WT), #p<0.05 (compared to Gclm^{+/-})

Figure 5.

ROS induces angiogenesis in Gclm^{+/-} mice through a VEGF dependent pathway. Panel A represents the VEGF level in ischemic tissues following induction of hind limb ischemia in Gclm WT, +/– and –/– mice, and after the addition of tempol in Gclm^{+/-}. Panel B shows blood flow data following induction of hind limb ischemia and the addition of either a VEGF₁₆₄ aptamer (VEGF₁₆₄ antagonist) or a heat denatured form of VEGF₁₆₄ aptamer. Panel C reports the quantitative measurement of the CD31:DAPI ratio calculated from images of ischemic tissues at day 7 following ligation and treatment with the VEGF₁₆₄ aptamer in Gclm^{+/-} mice. Panel D shows the quantitative measurement of the Ki67: DAPI ratio calculated from images of ischemic tissues at day 7 following ligation and treatment with either denatured VEGF₁₆₄ aptamer (DN-VEGF Aptamer) or VEGF₁₆₄ aptamer in Gclm^{+/-} mice. Panel E represents the VEGF level in ischemic tissues following induction of hind limb ischemia and treatment with the VEGF₁₆₄ aptamer in Gclm^{+/-} mice. n=5 per cohort, *p<0.05 (compared to WT), #p<0.05 (compared to Gclm^{+/-})

Figure 6.
Summary diagram of manuscript.