Published in final edited form as:

J Cardiovasc Pharmacol. 2016 June ; 67(6): 458–464. doi:10.1097/FJC.0000000000000381.

Endothelial Cell Redox Regulation of Ischemic Angiogenesis

Richard A. Cohen^{*}, Colin E. Murdoch, Yosuke Watanabe, Victoria M. Bolotina^{**}, Alicia M. Evangelista, Dagmar Haeussler, Melissa D. Smith, Yu Mei, XiaoYong Tong, Jingyan Han, Jessica B. Behring, Markus M. Bachschmid, and Reiko Matsui

^{**}Vascular Biology Section and Ion Channel and Calcium Signaling Unit, Department of Medicine, Boston University School of Medicine

Abstract

The endothelium produces and responds to reactive oxygen and nitrogen species (RONS), providing important redox regulation to the cardiovascular system in physiology and disease. In no other situation are RONS more critical than in the response to tissue ischemia. Here, tissue healing requires growth factor-mediated angiogenesis that is in part dependent on low levels of RONS, which paradoxically must overcome the damaging effects of high levels of RONS generated as a result of ischemia. While generation of endothelial cell RONS in hypoxia/reoxygenation is acknowledged, the mechanism for their role in angiogenesis is still poorly understood. During ischemia, the major low molecular weight thiol glutathione (GSH) reacts with RONS and protein cysteines, producing GSH-protein adducts. Recent data indicate that GSH adducts on certain proteins are essential to growth factor responses in endothelial cells. Genetic deletion of the enzyme glutaredoxin-1, which selectively removes GSH protein adducts, improves, while its overexpression impairs, revascularization of the ischemic hindlimb of mice. Ischemia-induced GSH adducts on specific cysteine residues of several proteins, including p65 NFkB and the sarcoplasmic reticulum calcium ATPase-2 (SERCA2), appear to promote ischemic angiogenesis. Identifying the specific proteins in the redox response to ischemia has provided therapeutic opportunities to improve clinical outcomes of ischemia.

Keywords

angiogenesis; redox; endothelium; glutathione; glutaredoxin-1; ischemia

Introduction

Reactive oxygen, *eg.* superoxide anion and hydrogen peroxide, and reactive nitrogen species, *eg.* nitric oxide and peroxynitrite, (collectively, RONS) are low molecular weight reactive species created as a result of metabolism in respiring cells. Elevated levels of RONS have been measured in countless studies of various cardiovascular and metabolic diseases, notably diabetes mellitus. In animal models, scavenging of the oxidants has proved to be of therapeutic benefit. When they are present at high levels or for prolonged times, one of the

^{*}Address for correspondence: Richard A. Cohen, MD, Director, Vascular Biology Section, 650 Albany Street, Boston, MA 02118, Tel: 617-638-7115, racohen@bu.edu.

most prevalent reasons for the adverse effects of RONS is the irreversible damage to proteins, requiring protein degradation and re-synthesis to return protein function. Such damage is evidenced by biologically irreversible post-translational chemical modifications. Although many such modifications have been identified, the one with most notoriety is nitrotyrosine¹ due to the early development of a specific antibody. Nitrotyrosine, and other irreversible oxidative post-translational modifications (OPTM), chlorotyrosine, and cysteine sulfonic acid (-SO₃H), are all recognized biomarkers of oxidant stress occurring in animal models and found in tissue and blood of human patients with cardiovascular disease^{2,3,4,5,6}. These biomarkers have been demonstrated on proteins whose functions are essential to cellular homeostasis, including albumin⁷, endothelial nitric oxide synthase (eNOS)⁸, prostacyclin synthase⁹, the sarcoplasmic-endoplasmic reticulum calcium ATPase-2 (SERCA2)^{10,11}, manganese superoxide dismutase¹², and p21Ras¹³. As an example, nitrotyrosine-modified proteins accumulate in endothelial cells of skeletal muscle in a commonly used mouse model of hindlimb ischemia^{14,15}. In this model, a one centimeter length of the femoral artery is ligated and excised, allowing serial measurements of blood flow and other parameters over several weeks, during which angiogenesis and revascularization return blood flow to near normal. As another example of irreversible protein oxidation within endothelial cells during hindlimb ischemia in the mouse, the reactive cysteine-674 thiol of SERCA2 is in part irreversibly oxidized to the sulfonic acid as revealed by a specific antibody (Figure 1).

Signaling in endothelial cells by thiol adducts

Lower levels of RONS are now recognized to be essential for cell signaling functions. For example, there is substantial evidence that nitric oxide (NO) is an endogenous mediator of growth factor-mediated angiogenesis¹⁶, and in therapeutic amounts, NO can provide substantial protection from the effects of ischemia¹⁷. Also, interference with normal functions of low-level RONS is a potential reason why application of non-specific scavenging antioxidants such as vitamin C and E have failed as therapies for diseases involving damage caused by high levels of RONS. These factors suggest that mammalian cells have developed mechanisms to utilize low levels of RONS, while protecting themselves from higher levels. While not exclusively so, the major signaling actions of RONS are mediated by reversible protein modifications on cysteine residues^{18,19,20}. Alterations in function are associated with multiple oxidative modifications of the thiol group (Figure 2). The primary targets include cysteines that are accessible on the protein surface, as well as those that are maintained in the chemically reactive thiolate anion state (-S⁻ rather than -SH) by nearby positively charged arginine and lysine amino acids. Although high concentrations of RONS widely oxidize cysteines and other amino acids on proteins, low levels of RONS affect these reactive cysteines more specifically. As an example, during growth factor initiated signaling in endothelial cells, the thiol of cysteine-674 of SERCA2 serves as the exclusive target for glutathione (GSH) adducts. Despite the fact that there are many other cysteine thiols on the exterior of the protein, a cysteine to serine mutation of this one thiol nearly eliminates all GSH adducts occurring on the protein during growth factor-induced angiogenesis²¹. NO is well documented to produce *S*-nitroso thiols (-SNO), and hydrogen peroxide to produce sulfenic acid (-SOH) modification of thiols^{22,23}. However, both of these

thiol modifications readily react with abundant intracellular GSH to form the more stable GSH adduct (-SSG). In response to direct challenge with various oxidants *in vitro*, or in particular in response to ischemia *in vivo*, large amounts of glutathione adducts appear on reactive cysteines. As an example, during the first 24 hours of ischemia, cysteine-674 of SERCA2 abundantly forms GSH adducts that persist for at least several days (Figure 3).

Glutaredoxin-1

Likely because of the chemical stability of GSH cysteine adducts, the cell has evolved several thiol reductase enzymes that can remove GSH adducts. While thioredoxins and peroxiredoxins are abundant thiol reductases, glutaredoxin-1 (Glx1) is the most selective in its ability to remove GSH protein adducts³. This 12 kiloDalton protein removes GSH adducts from a protein cysteine thiol by transferring the GSH adduct to one of its own reactive thiols on cysteine 22 or 25. Glx1 is then reduced by free GSH, completing the catalytic cycle (Figure 4). There is growing awareness of the importance of Glx1 as a physiological regulator^{24,34}. Our studies show that Glx1 regulates hindlimb ischemia, when the abundance of GSH adducts is high. The level of protein GSH adducts present in ischemic muscle and the restoration of blood flow during the two weeks following femoral artery excision was significantly enhanced in global Glx1 knockout mice³⁵, consistent with an essential role of GSH adducts in the response to ischemia. In contrast, GSH adducts in the ischemic muscle were decreased and restoration of blood flow was impaired in global Glx1 overexpressing transgenic mice (Figure 5)²⁸. Additionally, when compared to wild type mice, endothelial-specific overexpression of Glx1 impaired the recovery from ischemia, resulting in necrotic loss of toes³⁵. Together, these findings indicate a critical role for GSH adducts on endothelial cell proteins during the response to ischemia. Furthermore, these studies establish Glx1 as a critical regulator and potential therapeutic target to address in improving clinical outcomes of limb ischemia. (See the section, "Involvement of GSH adducts on specific proteins", below).

Redox regulation of cellular mechanisms of angiogenesis in endothelial cells

During a normal response to ischemia induced by femoral artery excision, the involvement of RONS is well established. Throughout the subsequent recovery of blood flow, collateral arteries grow in diameter to conduct blood flow around the blocked artery, and the numbers of capillaries increase within ischemic muscle tissue. The increase in number of capillaries, termed angiogenesis, requires endothelial cell proliferation and migration. The latter cellular processes are driven by increased production by the ischemic cells of growth factors, the most abundant of which is vascular endothelium growth factor (VEGF). In cultured endothelial cells, VEGF causes proliferation, migration, and capillary-like tube formation. These endothelial angiogenic responses require signaling elements that mediate the response to VEGF including VEGF receptor-2, phosphatidylinositol-3 kinase, Akt phosphorylation, endothelial nitric oxide synthase (eNOS) phosphorylation, and NO production. NO and eNOS have proven to be essential in the angiogenic response *in vivo*, as demonstrated by the fact that the eNOS knockout mouse has only minimal restoration of blood flow following

femoral artery ligation¹⁶. The activation of angiogenic behavior in cultured endothelial cells by VEGF can be mimicked by direct addition of micromolar concentrations of exogenous NO²¹.

Interestingly, NO does not work alone, but requires other RONS. First, intracellular superoxide anion is required, as demonstrated by the fact that overexpression of either superoxide dismutase 1 or 2 prevents both VEGF and NO-induced endothelial cell migration³⁶. Superoxide anion is also required *in vivo* for the restoration of hindlimb blood flow in ischemia as indicated by a subnormal return of blood flow in mice deficient in Nox2 (gp91phox), a component of NADPH oxidase,³⁷ a major source of superoxide anion. The actual chemical identity of the essential RONS for a normal endothelial response to VEGF is uncertain. It has been suggested that peroxynitrite (ONOO⁻), the reaction product of nitric oxide and superoxide, is the true signaling RONS based on inhibition of VEGF-induced angiogenic responses by a ONOO⁻ degradation catalyst³⁸. This also makes sense from the point of view that ONOO⁻ is a much more potent thiol oxidant. For example, low micromolar concentrations of ONOO⁻ induce protein GSH adducts in cell free systems on cysteine-674, the reactive thiol on SERCA2, much more effectively than do similar concentrations of nitric oxide alone³⁹.

NO and superoxide are not the only RONS required for angiogenesis, because scavenging of hydrogen peroxide (H₂O₂) by overexpression of catalase inhibits hindlimb ischemia-induced angiogenesis *in vivo*⁴⁰ and angiogenic endothelial cell responses to VEGF in culture^{36,39}. Indeed, low micromolar concentrations of H₂O₂ induce cell migration that mimics the response to VEGF³⁶. NADPH oxidase (Nox) 4 apparently provides the H₂O₂, as knockdown of its expression globally⁴¹ or selectively in endothelial cells⁴² *in vivo* inhibits the angiogenic response to ischemia and prevents the angiogenic responses to VEGF in cell culture. The interaction of NO, superoxide anion, and H₂O₂ is complex, but we found that H₂O₂ production by Nox4 may be upstream of superoxide anion production by Nox2³⁶. Namely, the angiogenic response to exogenous H₂O₂ or VEGF is prevented by overexpression of intracellular superoxide dismutase (directly removing the downstream mediator, superoxide) as well as knockdown of Nox2 (the producer of superoxide)³⁶. Despite the complexity, both H₂O₂ from Nox4 and superoxide anion from Nox2 appear to be required for NO to perform its angiogenic functions.

The requirements for nitric oxide, Nox4-derived H₂O₂, and Nox2-derived superoxide anion to achieve a normal angiogenic response to VEGF also extend to the ability of the growth factor to stimulate protein GSH adducts in endothelial cells in culture³⁶. This is also compatible with the previously noted suggestion that ONOO⁻ is essential for protein GSH adducts to form in sufficient abundance for VEGF signaling. Indeed, like the scavengers of RONS mentioned above, overexpression of Glrx1 prevents the accumulation of GSH protein adducts and completely prevents VEGF-induced angiogenic responses in cultured endothelial cells²¹.

Involvement of GSH adducts on specific proteins in the redox regulation of angiogenesis

It is clear from proteomics studies of endothelial cell proteins on which GSH adducts form during ischemia, that multiple, perhaps hundreds of, proteins are involved^{35,43}. This is as might be expected, given that ischemia results in high concentrations of RONS and hundreds of proteins have been identified to have reactive cysteines⁴⁴. While it is uncertain if and how all the proteins on which GSH adducts occur are involved in the angiogenic response, it is certain that several are essential.

GSH adducts on the cysteine-674 thiol of SERCA2 are one example of a mechanistically essential GSH adduct. First, GSH adducts form abundantly on the SERCA2 thiol during the first 3 days of ischemia (Figure 3). The importance of this cysteine was demonstrated in a heterozygote mouse in which one allele of SERCA2 was mutated to encode a serine in place of cysteine-674⁴⁵. Total GSH adducts on SERCA2 in ischemic muscle were decreased approximately 50%, consistent with this single thiol being the primary site for GSH adduct formation on SERCA2^{21,39}. In cultured endothelial cells, knockdown of SERCA2 expression prevented VEGF-induced calcium influx, cell migration, and capillary-like network formation. The importance of SERCA2 was attributed to its role in refilling intracellular calcium stores, which, in turn, regulates entry of the extracellular calcium required for angiogenic responses to VEGF. Overexpression of a SERCA2 cysteine-674 serine mutant or overexpression of Glrx1 in endothelial cells largely prevented increases in GSH adducts on SERCA2, calcium signaling, and angiogenic responses to VEGF. In addition, the mouse in which 50% of the redox-sensitive cysteines were mutated to serine showed impaired restoration of blood flow in the ischemic hindlimb (Figure 3). The fundamental importance of GSH adducts on cysteine-674 of SERCA2 for growth factor-mediated angiogenesis was also evidenced by the fact that the mouse with homozygote substitution of the SERCA2 serine mutation died *in utero* at the stage when heart and vascular development occur⁴⁵.

GSH protein adducts on any of several components involved in NFkB signaling inhibits activation of the pathway, i.e. by inactivating IKK²⁵ and limiting the DNA binding ability of p65⁴⁶ and p50⁴⁷. For example, increased GSH adducts were noted on p65 in normal ischemic hindlimb muscle and endothelial cells exposed to hypoxia, suggesting that during ischemia, NFkB activity is inhibited by glutathione adduct formation (Figure 5). On the contrary, NFkB activity is dramatically increased in endothelial cells cultured from transgenic mice overexpressing Glrx1²⁸. Overexpression of Glrx1 in endothelial cells prevented p65 GSH adducts and resulted in the activation of NFkB. A consequence of this activation was increased production of the non-canonical Wnt ligand, Wnt5a, levels of which rose dramatically in the ischemic hindlimbs of Glrx1 transgenic mice. Wnt5a, in turn, increased production of soluble Flt, an alternatively spliced VEGF receptor that acts as a decoy, interfering with binding and response to VEGF²⁸. Knocking down the expression of sFlt restored angiogenic function to endothelial cells that overexpressed Glrx1²⁸. Regulation and downstream targets of NF-kB are, however, complex. For example, mice with dominant negative Ikbα, in which NF-kB is inhibited, showed decreased blood flow recovery after

hindlimb ischemia⁴⁸. Induction of sFlt in Glrx1 overexpressing mice may result not only from NF- κ B activation but also other transcriptional controls regulated by GSH adducts.

Phosphatases (*eg.* protein tyrosine phosphatase-1B and protein phosphatase-2A) are protein targets that are inhibited by oxidants, resulting in increased phosphorylation and activation of kinase signaling. VEGF was shown to induce increases in GSH adducts on low molecular weight protein tyrosine phosphatases⁴⁹. In cultured endothelial cells, inhibition of the phosphatases by GSH adducts was required for increases in tyrosine phosphorylation and increased activity of focal adhesion kinase, a key mediator of endothelial cell migration. Either antioxidants or a ONOO⁻ decomposition catalyst were able to prevent GSH adducts from forming on the phosphatase, and thus prevented kinase activation. These studies indicate that endothelial cell migration requires GSH adducts and inhibition of the phosphatase to allow downstream kinase signaling.

p21ras is essential for angiogenesis. Glutathione adducts on cysteine 118 of p21ras substantially increase its activity⁵⁰, so it is possible that this small GTPase is another key protein that is redox-regulated by GSH adducts during ischemic angiogenesis. It is known that mutation of cysteine-118 in p21ras prevents a critical angiogenic role of eNOS in tumor maintenance⁵¹, making it likely that GSH adducts are involved.

Whereas GSH adducts on the above cited proteins would appear to aid in the restoration of blood flow during ischemia, GSH adducts on some proteins known to be essential in the angiogenic response to ischemia may have adverse consequences. For example, both eNOS¹⁶ and sirtuin-1⁵² are essential to normal angiogenesis and possess reactive cysteines that when adducted with GSH interfere with their function. Thus, GSH adducts on both eNOS³² and sirtuin-1⁵³ inhibit their catalytic activities, which would therefore be predicted to have a deleterious effect on ischemia-induced angiogenesis. As GSH adducts on both proteins are reduced by Glrx1^{54,32}, the deleterious effect of overexpression of Glrx1 itself cannot be explained. This apparent contradiction could exist for several reasons, including the possibility that these proteins are protected from GSH adducts during ischemia because of subcellular localization (*eg.* calveoli and nucleus, respectively), or that the global effects of up- or down-regulation of Glrx1 expression overrides changes in the function of these two proteins. It is also possible that because GSH adducts on eNOS uncouple the enzyme³², the increased ONOO⁻ that results may benefit VEGF signaling by further increasing GSH adducts.

Summary and future directions

It is now evident that GSH adducts form abundantly on proteins during ischemia, and that from studies of up- and down-regulation of Glrx1, formation of these adducts is important to the outcome of ischemia. It is possible that the benefit of GSH adducts on many proteins during ischemia is derived from protecting critical reactive cysteines against potential irreversible oxidation. The resulting preservation of cysteine function could explain the adverse consequences of overexpression and the benefit of knockdown of Glrx1. This then suggests that inhibition of Glrx1, because of its generalized effects on GSH adducts during ischemia, might be one therapeutic maneuver identified by these studies. It is also possible

to affect GSH adduct formation by influencing GSH levels and the redox state of GSH itself, which is regulated by GSH reductase and GSH synthase, amongst other factors.

There are several additional considerations that are pertinent to the interpretation of the abundance of GSH-adducted proteins during ischemia/reperfusion. (1) While many proteins may become decorated with GSH-adducts (perhaps serving as an oxidative buffer), few of these may be functionally altered by the modification; (2) Glrx1 likely displays different catalytic efficiencies for different proteins-GSH substrates; hence many protein-GSH substrates may be well below their K_m for deglutathionylation and therefore would be deglutathionylated much more slowly by Glrx1. These slowly reversible protein-GSH adducts would be less likely to serve as signaling intermediates. Unfortunately, very few studies of catalytic efficiency of Glrx1 for different protein-GSH substrates have been reported.

The global increase in GSH adducts during ischemia is difficult to reconcile with the apparent importance of the redox state of reactive cysteines in the function of individual proteins. The evidence, for instance, that GSH adducts on one cysteine of SERCA2 are essential to angiogenic growth factor signaling and that a 50% substitution of the cysteine inhibits ischemic angiogenesis *in vivo*, suggests that GSH adducts on some proteins might be more important than on others. If this is so, it might be possible to use the redox state of these particular cysteines, such as was measured for SERCA2 cysteine-674 with immunohistochemistry in Figure 1, as an indicator or biomarker of a favorable or unfavorable response to ischemia. Potentially, proteins have evolved with reactive cysteines, in part, for protection from ischemia, or other acute adverse redox conditions. This notion is supported by preliminary proteomic studies that we designed to identify which endothelial cell proteins have GSH adducts on reactive cysteines. Using systems biology approaches, these studies show that many proteins in several categories of tissue healing and angiogenesis have increased adducts during prolonged hypoxia, raising the possibility that protection of these categories of proteins in particular has evolved to improve the response to adverse oxidative stress conditions including ischemia. Future systems biology analysis might reveal that proteins with essential reactive cysteines work not alone, but in unison to accomplish a normal response to ischemia.

Acknowledgements

The work summarized in this review and writing of the manuscript were supported by funding from the National Institutes of Health grants RO1HL31607 and R37HL104017 (RAC), 5RO1HL54150 (VMB), RO1DK103750 (MMB), T32HL07969 (MDS), T32HL007501(JBB), T32HL07224, American Heart Association Scientist Development Grant 20140036, and the Boston University Clinical Translational Science Institute 1UL1TR001430 (J.H.).

References

1. MacMillan-Crow LA, Crow JP, Kerby JD, Beckman JS, Thompson JA. Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc.Natl.Acad.Sci.U.S.A.* 1996; 93(21):11853–11858. [PubMed: 8876227]
2. Cohen RA, Adachi T. Nitric-oxide-induced vasodilatation: regulation by physiologic S - glutathiolation and pathologic oxidation of the sarcoplasmic endoplasmic reticulum calcium ATPase. *Trends Cardiovasc.* 2006; 16(4):109–114.

3. Mieyal JJ, Chock PB. Posttranslational modification of cysteine in redox signaling and oxidative stress: Focus on S-glutathionylation. *Antioxid.Redox.Signal.* 2012; 16(6):471–475. [PubMed: 22136616]
4. Hong SJ, Gokulrangan G, Schoneich C. Proteomic analysis of age dependent nitration of rat cardiac proteins by solution isoelectric focusing coupled to nanoHPLC tandem mass spectrometry. *Exp.Gerontol.* 2007; 42(7):639–651. [PubMed: 17481840]
5. Stadtman ER, Van Remmen H, Richardson A, Wehr NB, Levine RL. Methionine oxidation and aging. *Biochim. Biophys. Acta - Proteins Proteomics.* 2005; 1703(2):135–140.
6. Shao B, Pennathur S, Heinecke JW. Myeloperoxidase targets apolipoprotein A-I, the major high density lipoprotein protein, for site-specific oxidation in human atherosclerotic lesions. *J. Biol. Chem.* 2012; 287(9):6375–6386. doi:10.1074/jbc.M111.337345. [PubMed: 22219194]
7. Odhiambo A, Perlman DH, Huang H, et al. Identification of oxidative post-translational modification of serum albumin in patients with idiopathic pulmonary arterial hypertension and pulmonary hypertension of sickle cell anemia. *Rapid Commun.Mass Spectrom.* 2007; 21(14):2195–2203. [PubMed: 17569101]
8. Xu J, Xie Z, Reece R, Pimental D, Zou MH. Uncoupling of endothelial nitric oxidase synthase by hypochlorous acid: role of NAD(P)H oxidase-derived superoxide and peroxynitrite. *Arterioscler.Thromb.Vasc.Biol.* 2006; 26(12):2688–2695. [PubMed: 17023679]
9. Nie H, liang Wu J, Zhang M, Xu J, Zou MH. Endothelial Nitric Oxide Synthase Dependent Tyrosine Nitration of Prostacyclin Synthase in Diabetes In Vivo. *Diabetes.* 2006; 55(11):3133–3141. [PubMed: 17065353]
10. Adachi T, Matsui R, Xu S, et al. Antioxidant improves smooth muscle sarco/endoplasmic reticulum Ca(2+)-ATPase function and lowers tyrosine nitration in hypercholesterolemia and improves nitric oxide-induced relaxation. *Circ.Res.* 2002; 90(10):1114–1121. [PubMed: 12039802]
11. Xu S, Ying J, Jiang B, et al. Detection of sequence-specific tyrosine nitration of manganese SOD and SERCA in cardiovascular disease and aging. *Am.J.Physiol Hear. Circ.Physiol.* 2006; 290(6):H2220–H2227.
12. Guo W, Adachi T, Matsui R, et al. Quantitative assessment of tyrosine nitration of manganese superoxide dismutase in angiotensin II-infused rat kidney. *Am.J.Physiol Hear. Circ.Physiol.* 2003; 285(4):H1396–H1403.
13. Zhao C, Sethuraman M, Clavreul N, Kaur P, Cohen RA, O'Connor PB. Detailed map of oxidative post-translational modifications of human p21ras using Fourier transform mass spectrometry. *Anal. Chem.* 2006; 78(14):5134–42. [PubMed: 16841939]
14. Turgeon J, Haddad P, Dussault S, et al. Protection against vascular aging in Nox2-deficient mice: Impact on endothelial progenitor cells and reparative neovascularization. *Atherosclerosis.* 2012; 223(1):122–9. [PubMed: 22658259]
15. Yan J, Tie G, Park B, Yan Y, Nowicki PT, Messina LM. Recovery from hind limb ischemia is less effective in type 2 than in type 1 diabetic mice: roles of endothelial nitric oxide synthase and endothelial progenitor cells. *J. Vasc. Surg.* 2009; 50(6):1412–22. [PubMed: 19837544]
16. Yu J, deMuinck ED, Zhuang Z, et al. Endothelial nitric oxide synthase is critical for ischemic remodeling, mural cell recruitment, and blood flow reserve. *Proc. Natl. Acad. Sci. USA.* 2005; 102(31):10999–11004. [PubMed: 16043715]
17. Xia Z, Vanhoutte PM. Nitric oxide and protection against cardiac ischemia. *Curr. Pharm. Des.* 2011; 17(18):1774–82. [PubMed: 21631419]
18. Winterbourn CC, Hampton MB. Thiol chemistry and specificity in redox signaling. *Free Radic. Biol. Med.* 2008; 45(5):549–561. [PubMed: 18544350]
19. Go Y-M, Jones DP. Thiol/disulfide redox states in signaling and sensing. *Crit. Rev. Biochem. Mol. Biol.* 2013; 48(2):173–81. [PubMed: 23356510]
20. Kumar V, Calamaras TD, Haeussler D, et al. Cardiovascular redox and ox stress proteomics. *Antioxid Redox Signal.* 2012; 17(11):1528–1559. [PubMed: 22607061]
21. Evangelista AM, Thompson MD, Weisbrod RM, et al. Redox regulation of SERCA2 is required for vascular endothelial growth factor-induced signaling and endothelial cell migration. *Antioxid Redox Signal.* 2012; 17(8):1099–1108. [PubMed: 22472004]

22. Yang Y, Loscalzo J. S-nitrosoprotein formation and localization in endothelial cells. *Proc.Natl.Acad.Sci.U.S.A.* 2005; 102(1):117–122. [PubMed: 15618409]
23. Saurin AT, Neubert H, Brennan JP, Eaton P. Widespread sulfenic acid formation in tissues in response to hydrogen peroxide. *Proc.Natl.Acad.Sci.U.S.A.* 2004; 101(52):17982–17987. [PubMed: 15604151]
24. Lillig CH, Berndt C. Glutaredoxins in thiol/disulfide exchange. *Antioxid. Redox Signal.* 2013; 18(13):1654–65. [PubMed: 23231445]
25. Reynaert NL, van der Vliet A, Guala AS, et al. Dynamic redox control of NF-kappaB through glutaredoxin-regulated S-glutathionylation of inhibitory kappaB kinase beta. *Proc Natl Acad Sci U S A.* 2006; 103(35):13086–13091. [PubMed: 16916935]
26. Shelton MD, Distler AM, Kern TS, Mieyal JJ. Glutaredoxin regulates autocrine and paracrine proinflammatory responses in retinal glial (müller) cells. *J Biol Chem.* 2009; 284(8):4760–4766. [PubMed: 19074435]
27. Gallogly MM, Starke DW, Mieyal JJ. Mechanistic and kinetic details of catalysis of thiol-disulfide exchange by glutaredoxins and potential mechanisms of regulation. *Antioxid Redox Signal.* 2009; 11(5):1059–1081. [PubMed: 19119916]
28. Murdoch CE, Shuler M, Haeussler DJ, et al. Glutaredoxin-1 up-regulation induces soluble vascular endothelial growth factor receptor 1, attenuating post-ischemia limb revascularization. *J. Biol. Chem.* 2014; 289(12):8633–8644. [PubMed: 24482236]
29. Gallogly MM, Shelton MD, Qanungo S, et al. Glutaredoxin regulates apoptosis in cardiomyocytes via NFkappaB targets Bcl-2 and Bcl-xL: implications for cardiac aging. *Antioxid Redox Signal.* 2010; 12(12):1339–1353. [PubMed: 19938943]
30. Pan S, Berk BC. Glutathiolation Regulates Tumor Necrosis Factor- α -Induced Caspase-3 Cleavage and Apoptosis: Key Role for Glutaredoxin in the Death Pathway. *Circ. Res.* 2007; 100(2):213–219. [PubMed: 17185628]
31. Okuda M, Inoue N, Azumi H, et al. Expression of Glutaredoxin in Human Coronary Arteries: Its Potential Role in Antioxidant Protection Against Atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* 2001; 21(9):1483–1487. [PubMed: 11557676]
32. Chen C-AA, De Pascali F, Basye A, Hemann C, Zweier JL. Redox modulation of endothelial nitric oxide synthase by glutaredoxin-1 through reversible oxidative post-translational modification. *Biochemistry.* 2013; 52(38):6712–6723. [PubMed: 23977830]
33. Bachschmid MM, Xu S, Maitland-Toolan KA, Ho YS, Cohen RA, Matsui R. Attenuated cardiovascular hypertrophy and oxidant generation in response to angiotensin II infusion in glutaredoxin-1 knockout mice. *Free Radic Biol Med.* 2010; 49(7):1221–1229. [PubMed: 20638471]
34. Wang J, Boja ES, Tan W, et al. Reversible glutathionylation regulates actin polymerization in A431 cells. *J Biol Chem.* 2001; 276(51):47763–47766. doi:10.1074/jbc.C100415200. [PubMed: 11684673]
35. Watanabe Y, Murdoch C, Han J, Cohen R, Mastui R. S-Glutathionylation of Endothelial Cell Proteins Facilitates Ischemic Limb Revascularization. *Circulation.* 2014; 130(Suppl 2):A16856.
36. Evangelista AM, Thompson MD, Bolotina VM, Tong X, Cohen RA. Nox4- and Nox2-dependent oxidant production is required for VEGF-induced SERCA cysteine-674 S-glutathiolation and endothelial cell migration. *Free Radic Biol Med.* 2012; 53(12):2327–2334. [PubMed: 23089226]
37. Tojo T, Ushio-Fukai M, Yamaoka-Tojo M, Ikeda S, Patrushev N, Alexander RW. Role of gp91phox (Nox2)-containing NAD(P)H oxidase in angiogenesis in response to hindlimb ischemia. *Circulation.* 2005; 111(18):2347–2355. [PubMed: 15867174]
38. El-Remessy AB, Al-Shabrawey M, Platt DH, et al. Peroxynitrite mediates VEGF's angiogenic signal and function via a nitration-independent mechanism in endothelial cells. *FASEB J.* 2007; 21(10):2528–2539. [PubMed: 17384142]
39. Adachi T, Weisbrod RM, Pimentel DR, et al. S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. A mechanism targeted by oxidants in vascular disease. *Nat.Med.* 2004; 10(11):1200–1207. [PubMed: 15489859]

40. Hodara R, Weiss D, Joseph G, et al. Overexpression of catalase in myeloid cells causes impaired postischemic neovascularization. *Arterioscler. Thromb. Vasc. Biol.* 2011; 31(10):2203–2209. [PubMed: 21799178]
41. Schroder K, Zhang M, Benkhoff S, et al. Nox4 is a protective reactive oxygen species generating vascular NADPH oxidase. *Circ.Res.* 2012; 110(9):1217–1225. [PubMed: 22456182]
42. Chen L, Xiao J, Kuroda J, et al. Both hydrogen peroxide and transforming growth factor beta 1 contribute to endothelial Nox4 mediated angiogenesis in endothelial Nox4 transgenic mouse lines. *Biochim. Biophys. Acta.* 2014; 1842(12):2489–99. Pt A. [PubMed: 25315297]
43. Behring JBB, Kumar V, Whelan SAA, et al. Does reversible cysteine oxidation link the Western diet to cardiac dysfunction? *FASEB J.* 2014; (5):1975–1987. Epub ahead of print. [PubMed: 24469991]
44. Sethuraman M, McComb ME, Huang H, et al. Isotope-coded affinity tag (ICAT) approach to redox proteomics: identification and quantitation of oxidant-sensitive cysteine thiols in complex protein mixtures. *J.Proteome.Res.* 2004; 3(6):1228–1233. [PubMed: 15595732]
45. Thompson MD, Mei Y, Weisbrod RM, et al. Glutathione adducts on sarcoplasmic/endoplasmic reticulum Ca²⁺ ATPase Cys-674 regulate endothelial cell calcium stores and angiogenic function as well as promote ischemic blood flow recovery. *J. Biol. Chem.* 2014; 289(29):19907–16. [PubMed: 24920669]
46. Qanungo S, Starke DW, Pai HV, Mieyal JJ, Nieminen AL. Glutathione supplementation potentiates hypoxic apoptosis by S-glutathionylation of p65-NFκappaB. *J Biol Chem.* 2007; 282(25):18427–18436. [PubMed: 17468103]
47. Pineda-Molina E, Klatt P, Vazquez J, et al. Glutathionylation of the p50 subunit of NF-κappaB: a mechanism for redox-induced inhibition of DNA binding. *Biochemistry.* 2001; 40(47):14134–14142. [PubMed: 11714266]
48. Tirziu D, Jaba IM, Yu P, et al. Endothelial nuclear factor-κB-dependent regulation of arteriogenesis and branching. *Circulation.* 2012; 126(22):2589–2600. [PubMed: 23091063]
49. Abdelsaid MA, El-Remessy AB. S-Glutathionylation of LMW-PTP regulates VEGF-mediated FAK activation and endothelial cell migration. *J. Cell Sci.* 2012
50. Clavreul N, Adachi T, Pimental DR, Ido Y, Schoneich C, Cohen RA. S-glutathiolation by peroxynitrite of p21ras at cysteine-118 mediates its direct activation and downstream signaling in endothelial cells. *FASEB J.* 2006; 20(3):518–520. [PubMed: 16415107]
51. Lim K-HH, Ancrile BB, Kashatus DF, Counter CM. Tumour maintenance is mediated by eNOS. *Nature.* 2008; 452(7187):646–649. [PubMed: 18344980]
52. Potente M, Ghaeni L, Baldessari D, et al. SIRT1 controls endothelial angiogenic functions during vascular growth. *Genes Dev.* 2007; 21(20):2644–2658. [PubMed: 17938244]
53. Zee RS, Yoo CB, Pimentel DR, et al. Redox regulation of sirtuin-1 by S-glutathiolation. *Antioxid Redox Signal.* 2010; 13(7):1023–1032. [PubMed: 20392170]
54. Shao D, Fry JL, Han J, et al. A redox-resistant sirtuin-1 mutant protects against hepatic metabolic and oxidant stress. *J Biol Chem.* 2014
55. Ying J, Sharov V, Xu S, et al. Cysteine-674 oxidation and degradation of sarcoplasmic reticulum Ca(2+) ATPase in diabetic pig aorta. *Free Radic Biol Med.* 2008; 45(6):756–762. [PubMed: 18590812]

Figure 1.

Immuno-histochemical staining of oxidation of SERCA2 cysteine-674 in mouse hindlimb after 3 weeks of ischemia. An antibody was produced against the irreversibly oxidized sulfonic acid in the SERCA2 sequence containing cysteine-674 and validated previously⁵⁵. Marked increase in staining is present in endothelial cells of capillaries within the chronically ischemic muscle compared to a sham-operated limb and represents accumulation of the oxidized protein thiol during ischemia. Magnification 10x.

Figure 2. Schematic representation of oxidative modifications of protein (P) cysteine thiols. The reactive thiolate anion exposed to RONS forms reversible intermediates, including -SNO and -SOH . These can be further oxidized to irreversible forms, sulfinic ($\text{-SO}_2\text{H}$) and sulfonic ($\text{-SO}_3\text{H}$) acids, or may react with free GSH to form GSH adducts. These adducts may be reversed by glutaredoxin-1 (Glr1), forming oxidized GSH (GSSG) in the process.

Figure 3.

GSH adducts on SERCA2 cysteine-674 affect the severity of mouse hindlimb ischemia. A. Western blots with a monoclonal antibody against GSH protein adducts of immunoprecipitated SERCA2 show increased adducts in ischemic (I) muscle compared to the non-ischemic (N) muscle of wildtype (WT) mice 3 days following induction of ischemia. By comparison, there are decreased adducts in the ischemic limbs of a mouse expressing a heterozygote knockin allele (SKI) expressing a SERCA2 serine-674. Results of studies like those in panel A are summarized in panel B. C. LASER Döppler image of the ischemic limbs of WT and SKI mice after 28 days of ischemia. The ischemic limb is shown on the right; non-ischemic on the left. Blood flow is inversely proportional to color wavelength, thus the yellow and red areas have the highest blood flow and dark blue, the least. The absence of red on the limb on the SKI left limb indicates decreased blood flow. D. LASER Döppler images obtained before (pre) and immediately post femoral artery excision, and following 7-28 days of recovery. Significantly impaired blood flow recovery was demonstrated at 21 and 28 days. Data from ⁴⁵.

Figure 4.

Schematic diagram showing the molecular structure and catalytic activity of glutaredoxin-1. The 12 kDa protein has a CXXC motif containing in the human protein, cysteines 22 and 25 shown at left. The scheme at right shows how the chemically reduced Glrx1 (two –SH groups) reacts with a protein GSH adduct, reducing the protein thiol in the process and taking on the GSH adduct. The oxidized glutaredoxin-1 then reacts with GSH to form GSSG, returning glutaredoxin-1 to the fully reduced form.

Figure 5.

Glutaredoxin-1 overexpression impairs hindlimb ischemia. A. LASER Döppler and photographic images of ischemic (right) hind limbs of WT mice and transgenic mice globally overexpressing glutaredoxin-1 (TG). A marked lack of blood flow and cyanosis is seen in the transgenic mouse. Results of measurements made over 14 days of recovery are shown in panel B and indicate the worsened prolonged ischemia associated with glutaredoxin-1 overexpression. Panel C summarizes results of studies to quantify reversible thiol oxidation of p65 NFkB in hindlimb muscle from WT and TG mice. Free protein thiols in muscle lysates were chemically blocked, and then reversibly oxidized thiols were reduced and labeled with a biotin containing thiol tag. Pull-down of biotin-labeled proteins on streptavidin was followed by gel electrophoresis and immuno-blotting for p65. Ischemia caused a 7-fold increase in reversible thiol oxidation on p65 in muscle from WT mice, but no significant increase in TG mice overexpressing glutaredoxin-1. The presumption being made is that glutaredoxin-1 reduced GSH adducts on p65 thiols despite ischemic conditions. Panel D shows increased plasma levels of sFlt in TG mice at 4 and 14 days post-ischemia. Cell studies showed that sFlt, an antagonist of endothelial VEGF receptor-mediated signaling and angiogenic behavior, is normally suppressed during ischemia by GSH adducts on p65 that inhibit NFkB activity and Wnt5A-mediated sFlt production. In glutaredoxin-1 TG mice, fewer GSH adducts formed, and NFkB-dependent Wnt5A-mediated sFlt increased to explain impaired angiogenesis. From reference²⁸.