Endothelial Cell Redox Regulation of Ischemic Angiogenesis

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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 (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, (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, and in animal models, scavenging of the oxidants has therapeutic benefits. When they are present at high levels or for prolonged times, one of the 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 nitrotyrosine[1] due to the early development of a specific antibody. Nitrotyrosine, and other irreversible oxidative post-translational modifications (OPTM), chlorotyrosine, methionine sulfoxide, and cysteine sulfonic acid (-SO_3H), 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 albumin[7], endothelial nitric oxide synthase (eNOS)[8], prostacyclin synthase[9], the sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA)[10,11] , manganese superoxide dismutase[12], and p21Ras[13]. 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 angiogenesis[16], and in therapeutic amounts, NO can provide

substantial protection from the effects of ischemia[17]. 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 angiogenesis[21]. 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 peroxidoxins are abundant thiol reductases, glutaredoxin-1 (Glrx1) is the most selective in its ability to remove GSH protein adducts[3]. 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 23 or 26. Glrx1 is then reduced by free GSH, completing the catalytic cycle (Figure 4). There is growing awareness of the importance of Glrx1 as a physiological regulator [24–34]. Our studies show that Glrx1 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 Grx1 knockout mice[35], 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 Glrx1 overexpressing transgenic mice (Figure 5)[28]. Additionally, when compared to wild type mice, endothelial-specific overexpression of Glrx1 impaired the recovery from ischemia, resulting in necrotic loss of toes[35]., Together, these findings indicate a critical role for GSH adducts on endothelial cell proteins during the response to ischemia. Furthermore, these studies establish Grx1 as a critical regulator and potential therapeutic target to address in improving clinical outcomes of limb ischemia.

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 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 ligation[16]. The activation of angiogenic behavior in cultured endothelial cells by VEGF can be mimicked by direct addition of micromolar concentrations of exogenous NO[21].

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 migration[36]. 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 catalyst[38]. 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 alone[39].

NO and superoxide are not the only RONS required for angiogenesis, because scavenging of hydrogen peroxide (H_2O_2) 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 H_2O_2 induce cell migration that mimics

the response to VEGF[36]. NADPH oxidase (Nox) 4 apparently provides the H_2O_2, as knockdown of its expression globally[41] or selectively in endothelial cells[42] *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 H_2O_2 is complex, but we found that H_2O_2 production by Nox4 is upstream of superoxide anion production by Nox2[36]. Namely, the angiogenic response to exogenous H_2O_2 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]. Thus, both H_2O_2 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 H_2O_2, 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 culture[36]. 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 formation of GSH protein adducts and completely prevents VEGF-induced angiogenic responses in cultured endothelial cells[21].

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 cysteines [44]. 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-674[45]. 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 occur[45].

GSH protein adducts on any of several components involved in NFkB signaling inhibits activation of the pathway, i.e. by inactivating IKK[25] and limiting the DNA binding ability of p65[46] and p50[47]. 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 Glrx1[28]. 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 VEGF[28]. Knocking down the expression of sFlt restored angiogenic function to endothelial cells that overexpressed Glrx1[28]. Regulation and downstream targets of NF-kB are, however, complex. However, mice with dominant negative IκBα, in which NF-kB is inhibited, showed decreased blood flow recovery after hindlimb ischemia[48]. Induction of sFlt in Glrx1 overexpressing mice may result not only from NF-kB 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 phosphatases[49]. 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 activity[50], 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 maintenance [51], 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 eNOS[16] and sirtuin-1[52] are essential to normal angiogenesis and possess reactive cysteines that when adducted with GSH affect their function. However, GSH adducts on both eNOS[32] and sirtuin-1[53] 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 Grx1[54,32], the deleterious effect of Grx1 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 Grx1 expression overrides changes in the function of these two proteins. It is also possible that because GSH adducts on eNOS uncouple the enzyme[32], 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 Grx1, 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 Grx1. This then suggests that inhibition of Grx1, 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.

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 SERCA 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 SERCA 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.

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Figure legends:

1. Immuno-histochemical staining of oxidation of SERCA 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 previously[55]. 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.
2. Schematic representation of oxidative modifications of protein cysteine thiols. The reactive thiolate anion exposed to RONS forms reversible intermediates, -SNO and –SOH. These can be further oxidized to irreversible forms, sulfinic ($-SO_2H$) and sulfonic ($-SO_3H$) acids, or may react with free GSH to form GSH adducts. These adducts may be reversed by glutaredoxin-1 (Glrx1), forming oxidized GSH (GSSG) in the process.
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 immuno-precipitated 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. 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].
4. Schematic diagram showing the molecular structure and catalytic activity of glutaredoxin-1. The 10 kDa protein has a CXXC motif containing cysteines 23 and 26 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.

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 reference[28].

Figure 1
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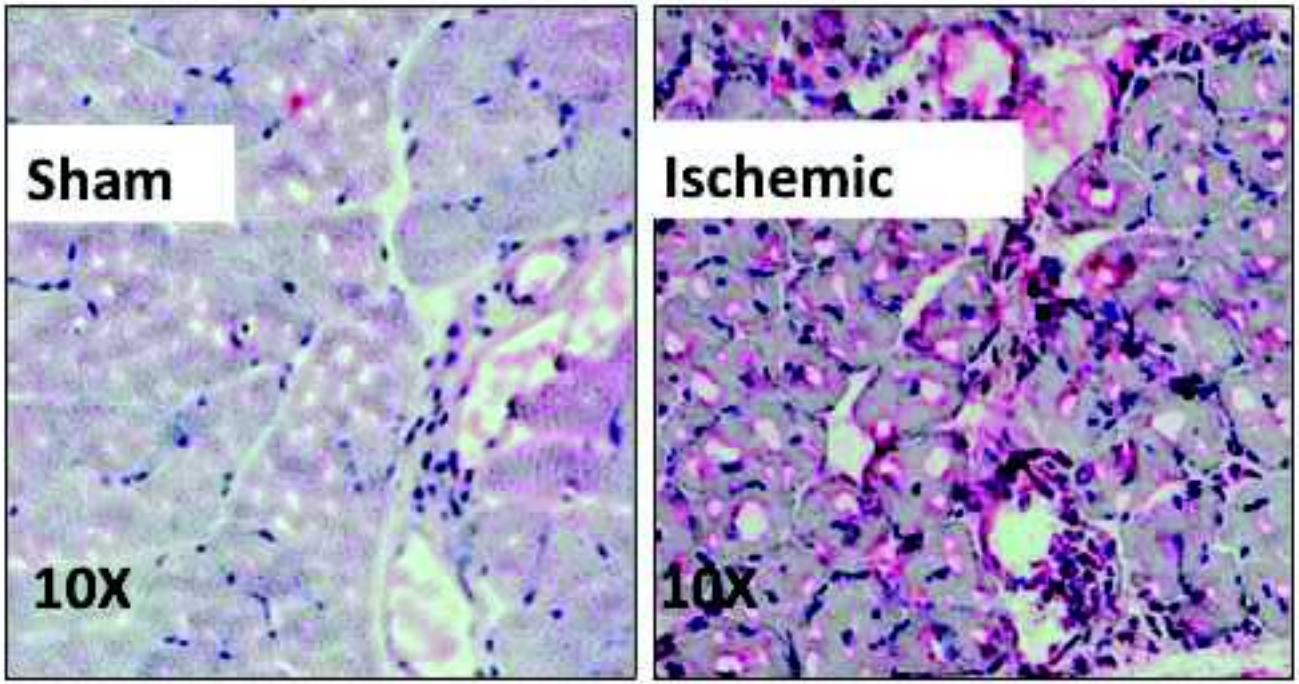

Figure 1

Figure 2
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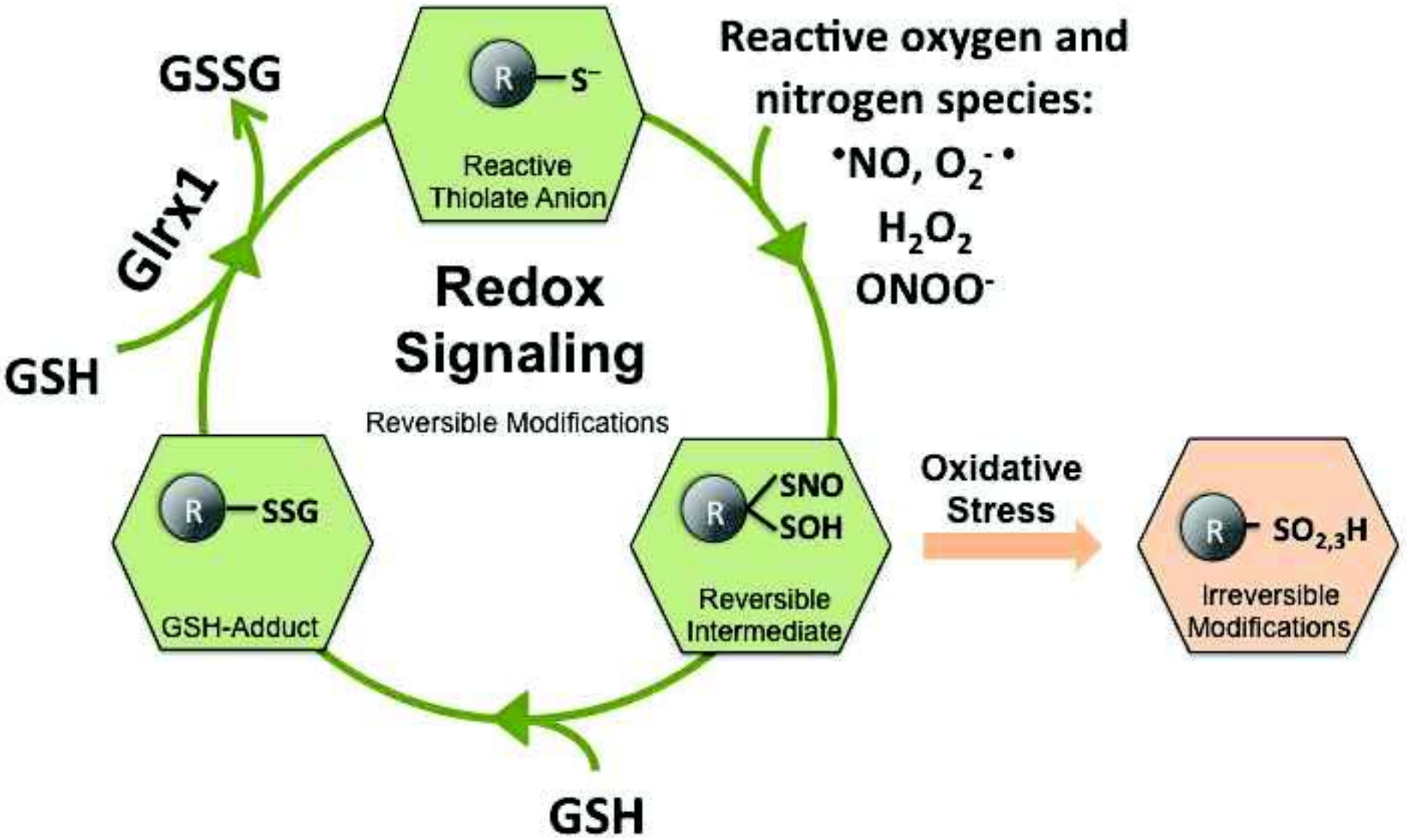

Figure 2

Figure 3
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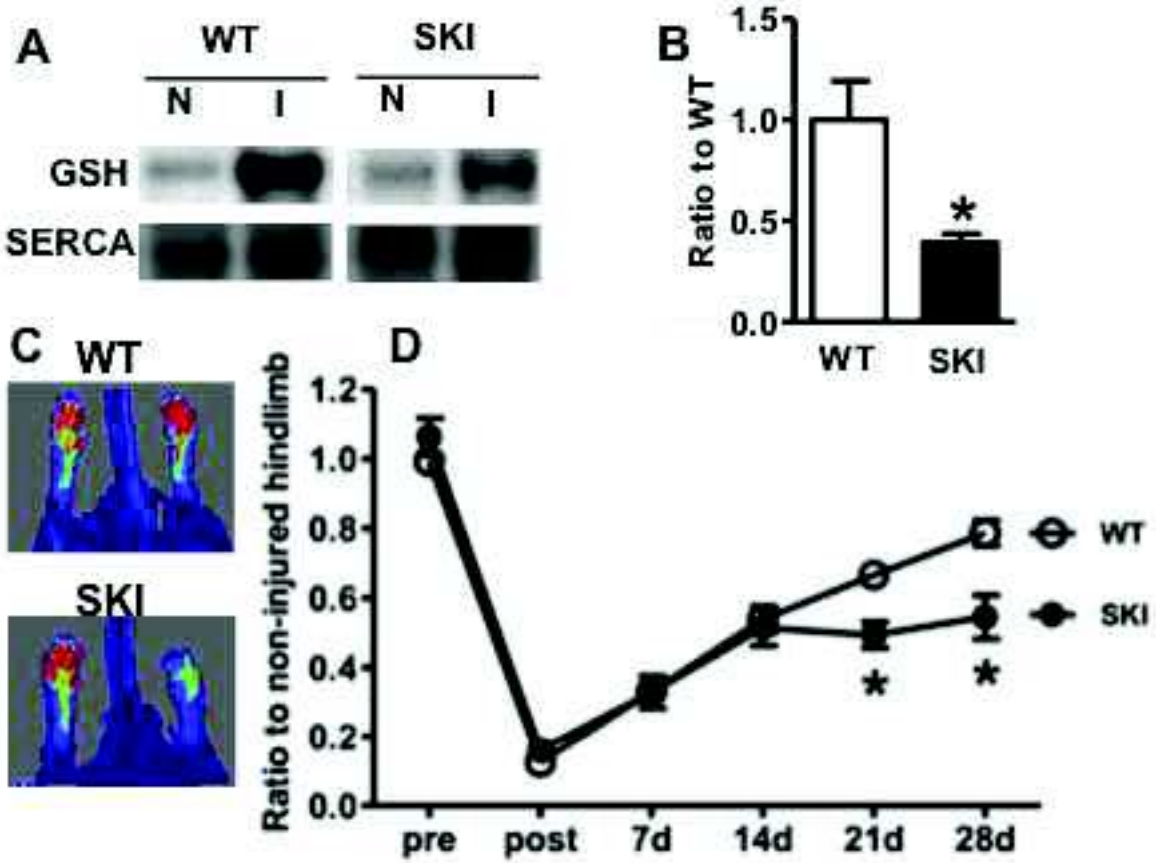

Figure 3

Figure 4
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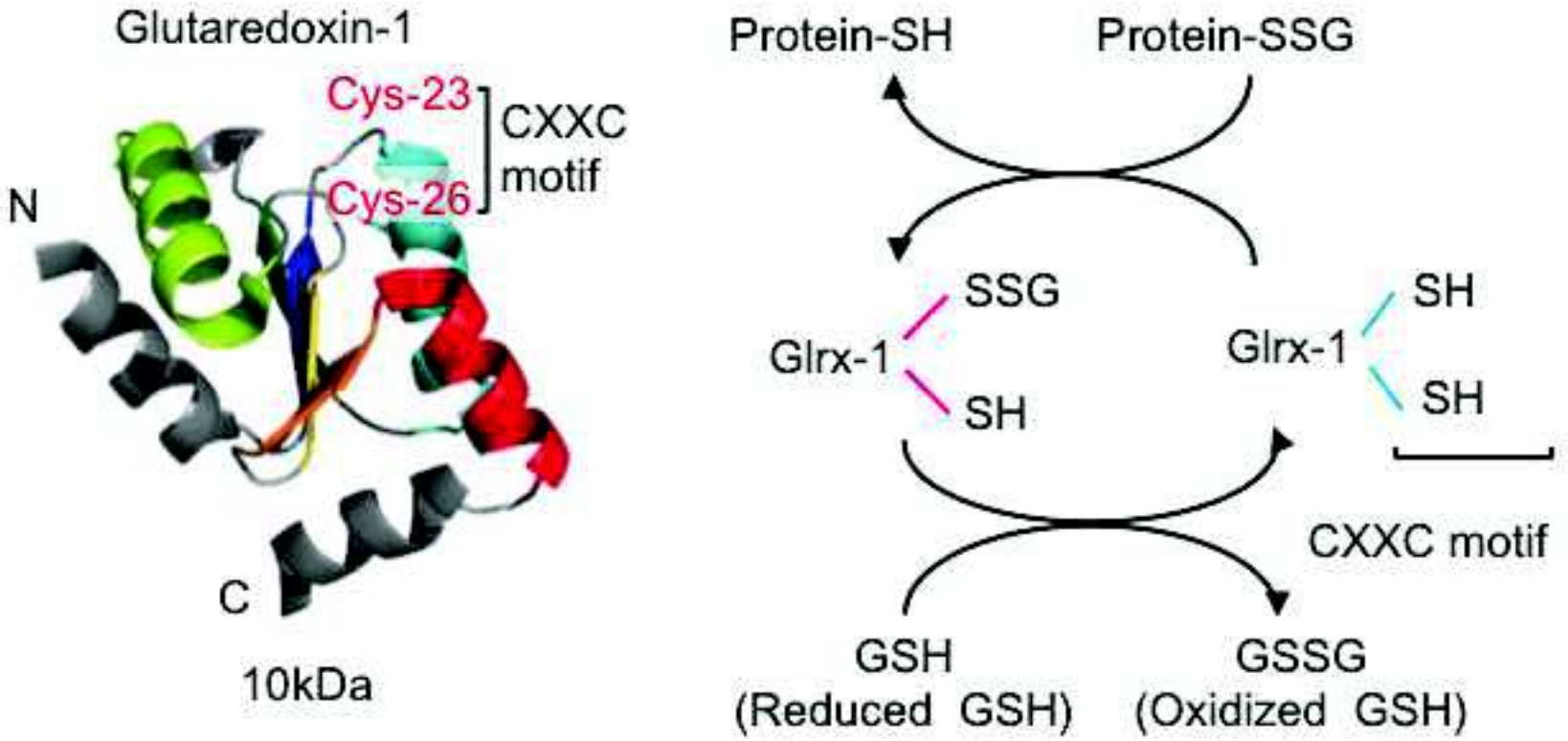

Figure 4

Figure 5
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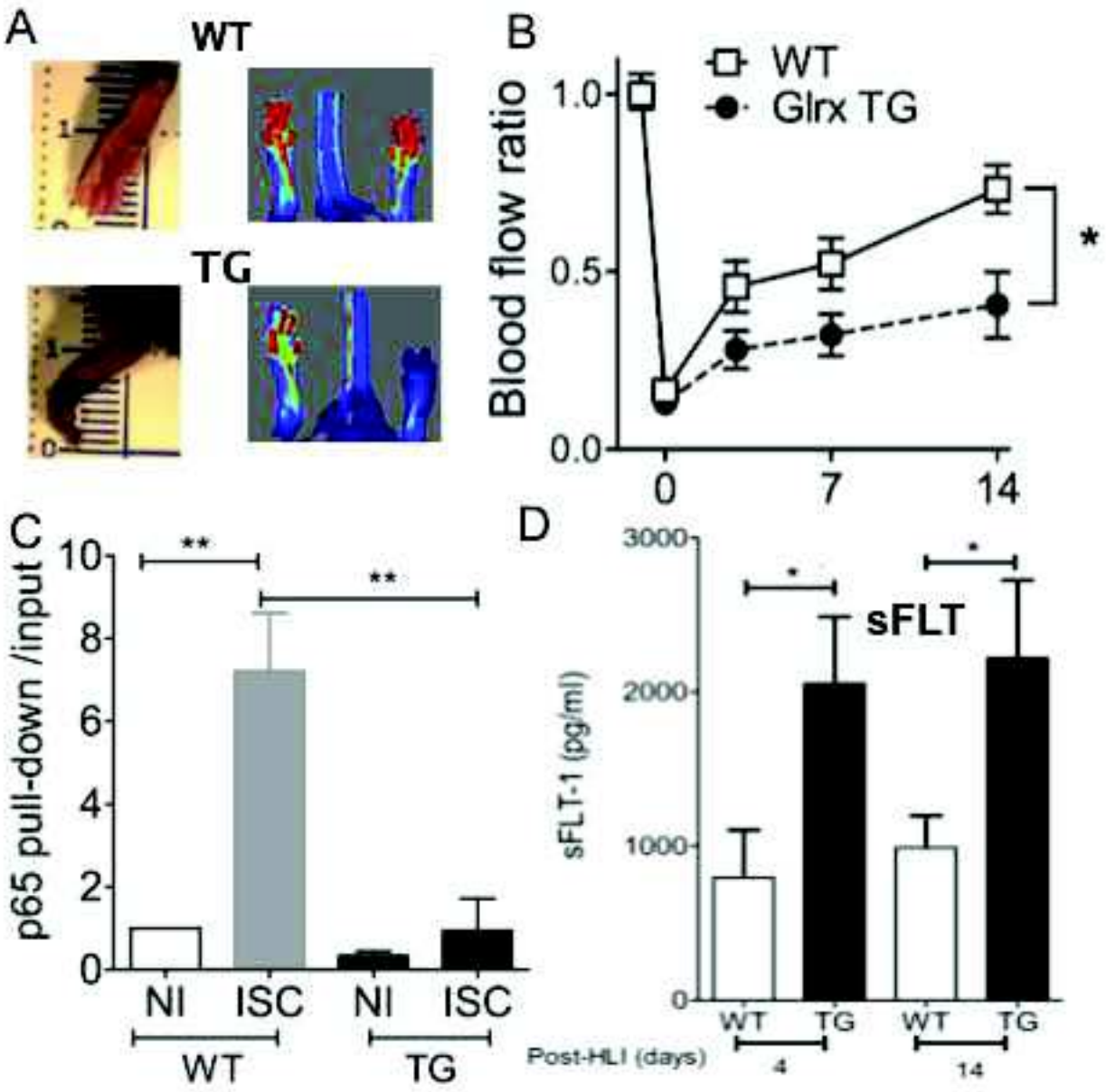

Figure 5