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Redox Balance Dynamically Regulates Vascular Growth and Remodeling

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Abstract

Vascular growth and remodeling responses entail several complex biochemical, molecular and cellular responses centered primarily on endothelial cell activation and function. Recent studies reveal that changes in endothelial cell redox status critically influence numerous cellular events that are important for vascular growth under different conditions. It has been known for some time that oxidative stress actively participates in many aspects of angiogenesis and vascular remodeling. Initial studies in this field were largely exploratory with minimal insight into specific molecular mechanisms and how these responses could be regulated. However, It is now clear that intracellular redox mechanisms involving hypoxia, NADPH oxidases (NOX), xanthine oxidase (XO), nitric oxide and its synthases, and intracellular antioxidant defense pathways collectively orchestrate a redox balance system whereby reactive oxygen and nitrogen species integrate cues controlling vascular growth and remodeling. In this review, we discuss key redox regulation pathways that are centrally important for vascular growth in tissue health and disease. Important unresolved questions and issues are also addressed that requires future investigation.

Keywords

hydrogen peroxide; superoxide; nitric oxide; antioxidant; angiogenesis

A. Introduction

Vascular growth and remodeling responses are critically important for all animal species. Importantly, regulation of vascular growth contributes to both normal physiological responses and numerous pathological disease processes. This is accomplished through differential vascular growth involving angiogenesis (increased microvascular growth), arteriogenesis (collateral artery remodeling and growth), and vasculogenesis (developmental and stem cell influenced growth). Significant insight has been made regarding how these events of vascular growth and remodeling are regulated at the cellular and molecular level through identification of critical cytokines (VEGF, bFGF, SDF-1, and others), signaling mediators (PKC, PI3 kinase, Akt, ERK 1/2, and others), and various cellular responses such as activation, proliferation, migration and maturation [1, 2]. However, all of these pathways

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and mediators are also significantly influenced by redox biology and biochemistry, of which the mechanistic roles of reactive chemical species, such as reactive oxygen species (ROS) or reactive nitrogen species (RNS) still remains poorly understood.

Figure 1 illustrates a model paradigm whereby the amount of redox stress, as defined by increased ROS/RNS production or loss of antioxidant responses, influences tissue vascular growth that is involved in both physiological and pathological responses. While not a universal phenomena, numerous experimental studies indicate that small amounts of redox stress significantly influence physiological vascular growth responses; whereas, greater amounts of oxidative or nitrosative stress contribute to vascular growth and remodeling under pathological conditions. In this review, we discuss important previous findings as well as highlight recent progress and understanding of redox regulation of vascular growth and remodeling. Due to space limitations, we are unable to discuss all findings in this field but have sought to guide the reader to additional information sources when necessary.

B. Sources of reactive oxygen and nitrogen species and their vascular compartments

Numerous intracellular redox regulators are produced within multiple cell types of the vasculature. Importantly, these regulators of vascular cell redox status exist in various forms, such as oxidants, free radicals, gasotransmitters, and various poly- or small peptide formats. In several ways, these molecules may both augment or attenuate the effects of the other depending on cell type, vascular health status, and functional vascular responses (Figure 2). Below we discuss some of the key mediators of redox regulation during vascular remodeling along with relevance to cell type.

NADPH oxidases (NOX)

An important source of ROS produced in vascular cells such as endothelial cells, vascular smooth muscle cells etc. comes from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, an enzyme which is responsible for the production of superoxide ($O_2^{\bullet-}$) using oxygen and NADPH as catalysts [3, 4]. The NADPH oxidase family consists of five isoforms (NOX1-5) including Duox 1 and 2 with mRNA of NOX1, NOX2, NOX4 and NOX5 expressed in the endothelium [5]. In general, P22phox is essential to form a complex with NOX1, NOX2, or NOX4 for initiation of enzyme activity [6].

NOX2 has six transmembrane domains where COOH and NH₂ terminus facing towards cytoplasm. NOX 2 is constitutively associated with p22phox and it is unstable in the absence of p22 phox [7]. NOX2 is located with the cytoskeleton, lipid raft/ caveolae and the perinuclear compartment; and requires association with p47 phox, p67 phox and Rac1/2 for its full activation. Once assembled, the complex is active and generates $O_2^{\bullet-}$ by transferring an electron from NADPH in the cytosol to oxygen on the luminal or extracellular space [8]. NOX1 localizes with p22phox in caveolae/lipid rafts and is expressed at low levels under physiological conditions and is activated in a similar way as NOX2 [9]. NOX1 and 2 are inactive in resting, unstimulated cells but their expression and activation are increased in response to different stimuli to generate ROS [10, 11].

NOX4 is located within the endoplasmic reticulum, mitochondria and nucleus of the endothelial cell; however, some uncertainty remains with respect to the importance of these various intracellular compartments for cellular responses. NOX4 is a dominant isoform within endothelial cells and believed to maintain basal ROS (H_2O_2 and $\text{O}_2^{\bullet-}$) generation [12, 13]. Importantly, p22phox, NOXR1 and Poldip2 bind to NOX4 and are required for its activation [14]. Although Rac is not required for p22phox dependent NOX4 enzyme activity, angiotensin II and high glucose stimulation increases NOX4 activity through a Rac 1 dependent pathway [15]. NOX4 is unique in that it may produce $\text{O}_2^{\bullet-}$ involving the B loop similar to other NOX isoforms but can also produce constitutive H_2O_2 through the E loop [10, 16]. NOX5 is also unique in that it possesses an amino terminal calmodulin like domain with four binding sites for calcium. Unlike, NOX1-4, NOX5 does not require p22phox for its activation. NOX5 is directly regulated by intracellular calcium, the binding of which causes conformational change leading to enhanced ROS formation [17].

NOX 1, NOX 2, NOX4 subunit expression and/or activity are increased in disease conditions such as diabetes, atherosclerosis and angiogenesis [14]. NOX enzymatic activity is also found in cultured vascular smooth muscle cells and functions in a similar manner to endothelial cells. NOX1 is located in the endoplasmic reticulum and membrane compartments of VSMC and PDGF, angiotensin II and $\text{PGF}_2\alpha$ can all increase NOX1 expression in VSMC [18]. Importantly, NOX1 activity can also be regulated by the redox chaperone protein disulfide isomerase in vascular smooth muscle cells [19]. NOX2 is found to colocalize with the perinuclear cytoskeleton in vascular smooth muscle cell (VSMC) [20] and its expression is induced by interferon- γ and angiotensin II. NOX4 is expressed in the endoplasmic reticulum and nucleus of the VSMC with ER stress, shear stress, hypoxia, ischemia, TNF- α , and TGF- β 1 regulating its expression [19]. Finally, NOX1-4 expression can also be found in the adventitia of vascular tissue with NOX 2 and 4 being predominant in fibroblasts and macrophages, respectively

NOX enzyme activity has been reported to play an important role in the regulation of angiogenic growth factors such as VEGF [21]. Hypoxia induced activation of NOX activity blocked by pharmacological inhibitors decreases ROS formation and also reduces VEGF production, which blunts Akt and ERK1/2 activation and subsequent vascular growth [21, 22]. Angiopoietin also stimulates NOX mediated ROS production and capillary tube formation contributing to angiogenic activity [23]. Thus, modulation of NOX enzyme expression and activity serves critical roles in regulating vascular growth and remodeling.

Mitochondrial Respiratory Chain

The mitochondrial respiratory chain is one of the largest sources of ROS in most mammalian cells [24]. Electron transport across the mitochondrial inner membrane is a primary site of ROS production within mitochondria. This is due to the function of complex I NADPH-ubiquinone oxidoreductase accepting electrons from NADH, which then transfers electrons to complex II continuing across an electrochemical gradient to complex III and finally to complex IV where they are used to reduce molecular oxygen to water [24]. Although the majority of oxygen is reduced to water under physiological conditions, about

1-4% is reduced to $O_2^{\bullet-}$ with the primary locations of $O_2^{\bullet-}$ production in the mitochondria at complexes I and III [24].

During tissue ischemia or cellular hypoxia, the electron transport process becomes uncoupled, leading to increased $O_2^{\bullet-}$ production resulting in numerous cellular responses. Excessive amount of ROS in mitochondria have been associated with pro-angiogenic stimulation. For example, inhibiting ROS production by mitochondria complex 1 using rotenone was found to inhibit the expression of VEGF and VEGF induced vascular growth and remodeling *in vivo* and *in vitro* [22]. However, much less information is known about how mitochondria and its associated ROS generation contribute to vascular growth and remodeling.

Nitric oxide (NO) synthesis and eNOS uncoupling

NO is a well-known gasotransmitter that stimulates vascular growth and remodeling. NO is synthesized by three isoforms of nitric oxide synthase (NOS) including NOS1 (inducible NOS/iNOS), NOS2 (neuronal NOS/nNOS), and NOS 3 (endothelial NOS/eNOS). These NOS isoforms are responsible for producing NO by sequential oxidation/reduction of L-arginine to L-citrulline [8]. The process of NO production varies within different vascular cell types. In endothelial cells, calcium- calmodulin dependent eNOS constitutively produces NO [25, 26] Conversely, iNOS produces NO from L-arginine in vascular smooth muscle cells in a calcium independent manner [27]. Whereas, NO may be produced in both constitutive and inducible manner within endothelium in response to angiogenic stimuli [28–30].

NO mediates cellular survival by inhibiting apoptosis while promoting proliferation within endothelial cells. Studies demonstrate that VEGF induced NO leads to the production of tube like structures in endothelial cells, which is blunted by the NOS antagonist N-nitro-L-arginine methylester (L-NAME) highlighting the importance of NO for VEGF dependent vascular growth and remodeling [31, 32]. It has been shown that eNOS is essential for angiogenic stimulation in the matrigel plug assay and tissue ischemia models using eNOS knock out mice [33, 34, 35]. However, NO can react with $O_2^{\bullet-}$ in a diffusion-limited manner to form peroxynitrite, a highly potent oxidizing and nitrosating agent that can uncouple eNOS and decrease NO production and bioavailability. Decreased substrate bioavailability (BH4 or L-arginine) also contributes to eNOS uncoupling resulting in electron transfer from the heme domain reducing oxygen to form $O_2^{\bullet-}$. Increased vascular $O_2^{\bullet-}$ production can further impair endothelium-dependent vascular relaxation through progressive interaction with NO resulting in further peroxynitrite generation. Moreover, peroxynitrite can oxidize BH4 to BH2, which in turn further perpetuates eNOS uncoupling [36]. Finally, NOX activity participates in BH4 oxidation that does not occur in p47phox^{-/-} mice highlighting the contribution of NOX for uncoupled eNOS production of ROS [37]. Together, these findings clearly indicate that generation of both ROS and RNS can operatively work to stimulate a vicious cycle of redox imbalance leading to vascular dysfunction.

Xanthine Oxidase (XO)

Xanthine oxidoreductase is another important source of ROS production in vascular cells. Xanthine oxidoreductase protein expression can exist in two forms including xanthine dehydrogenase (XDH) and xanthine oxidase (XO) [38]. XDH produces NADH and uric acid from hypoxanthine and xanthine, whereas XO uses oxygen as an electron acceptor from the xanthine and hypoxanthine to form $O_2^{\bullet-}$ and H_2O_2 [39, 40]. XO activation is induced by inflammatory cytokines and hypoxia which is mediated in part by NOX mediated H_2O_2 [41]. NOX augments endothelial cell XO activation that can modulate ROS production in response to oscillatory shear stress of endothelial cells [42]. Evidence also demonstrates that pharmacological XO inhibition attenuates $O_2^{\bullet-}$ production in both arteries and veins [43]. Importantly, a recent article by Kelley et al reports that under low oxygen tension (1% O_2) H_2O_2 is the major ROS produced by XO and under higher O_2 tensions (10-21% O_2) $O_2^{\bullet-}$ production is favored [44]. XO plays a context dependent important role in endothelial cell survival signaling which depends on the amount of ROS produced, such that low amount ROS ($O_2^{\bullet-}$ and H_2O_2) may act as physiological second messengers promoting vascular growth [45, 46]. Conversely, overproduction of ROS by XO may impair vascular endothelial function and growth [47]. In summary, it is clear that ROS has both positive and negative regulatory roles in vascular growth and remodeling. However, the precise concentrations and species of redox chemical mediators necessary for these positive and negative modulatory effects remain poorly defined.

C. Redox regulation of angiogenic signaling pathways and mediators

Angiogenesis is a physiological phenomenon involving multiple signaling and molecular processes that increases microvascular density. Angiogenesis processes such as vascular sprouting, remodeling and recruitment of endothelial cells are triggered by numerous angiogenesis stimulators, such as vascular endothelial growth factor (VEGF) and other cytokines, as well as inflammatory responses [48–50]. Reactive oxygen species (ROS) are implicated in regulating processes that involve endothelial cell activation, inflammation, vascular remodeling and angiogenesis [51–53]. It is well known that ROS such as $O_2^{\bullet-}$ and H_2O_2 are pro-inflammatory, resulting in intimal-medial thickness and considered as risk factors for vasculature pathology. However, ROS production regulates numerous signaling cascades (Figure 3) that facilitate angiogenesis activity [16, 54, 55]. Despite a plethora of information available in the literature involving redox signaling, their precise implications on angiogenesis signaling are less well defined.

ROS serve multiple roles in both intracellular signaling and molecular gene expression events influencing cellular protection or injury. Evidence suggests that a controlled redox balance is beneficial and induces signaling pathways promoting physiological angiogenesis contributing to cellular and tissue homeostasis; but if disturbed results in adverse effects on cellular and tissue function resulting in tissue pathology [56].

ROS can regulate downstream signaling mediators of angiogenesis, which may occur in several ways such as: 1) direct modulation of signal molecules involved in angiogenesis such as cytokine receptors, 2) modulation of intermediary regulatory molecules of angiogenesis such as phosphatase, kinase, and/or cofactors, and 3) modification of

transcription factors that affect signaling molecule and cell survival gene expression [57]. However, it is not appropriate to conclude that ROS are predominantly harmful by-products of metabolism considering its role as a critical signaling mediator in numerous cellular functions.

ROS and VEGF signaling pathways

VEGF is a primary angiogenic cytokine that has gained prominence as a major inducer of angiogenesis and vasculogenesis under physiological and pathological conditions [58]. VEGF activates several endothelial cell responses through VEGFR-1/Flt-1 and VEGFR-2/Flk-1/KDR that are necessary for normal vascular development [59, 60]. Stimulation of VEGFR-1/Flt-1 has been implicated in EC migration; whereas, 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 (PLC) [61–64].

It is clear from the literature that angiogenic cytokine mediated ROS production, such as $O_2^{\bullet-}$ and H_2O_2 is important for several aspects of angiogenesis activity [56, 65, 66]. VEGF dependent signaling stimulates the production of ROS in endothelial cells that is mediated through NOX activation [65]. NOX activation may also occur in response to angiopoietin-1 as well as hypoxia and ischemia [21, 67]. Recently Garrido-Urbani et al has reported that genetic deficiency of NOX1, but not NOX2 or 4, decreases angiogenic activity in mice [68]. Specifically, NOX1 knockout mice were found to have decreased matrigel plug and tumor angiogenesis responses along with decreased endothelial cell migration and tube formation in vitro involving inhibition of the NF- κ B regulator peroxisome proliferator-activated receptor α (PPAR α). Contrary to the notion that NOX4 decreases angiogenic activity, Craige et al has demonstrated in NOX4 overexpressor mice model augmented angiogenesis under ischemia conditions [69].

Recently it has been shown that overexpression of NOX4 significantly enhances VEGF-induced ROS production that is involved in VEGFR2 autophosphorylation and Akt activation, thereby promoting EC migration and proliferation, which was attenuated after enzyme knockdown using NOX4-siRNA [70, 71]. Moreover, Nox4 mediated production of H_2O_2 activates ERK1/2 to promote endothelial cell proliferation, which is also inhibited upon downregulation of NOX4 protein expression using siRNA [72].

ROS and phosphatase activity

Phosphorylation of signaling proteins is controlled by multiple kinases, which are in turn regulated by families of protein phosphatases. Phosphatase enzyme activity is essential for removing phosphate groups from phospho-kinases resulting in either kinase activation or inactivation. As such, phosphatase activity critically regulates numerous intracellular signaling pathways [73]. Importantly, protein phosphatase activity is regulated by the reversible oxidation of their catalytic Cys residues by H_2O_2 which attenuates protein dephosphorylation thereby facilitating signal transduction [74]. Protein phosphatases are protected by cysteinyl S-nitrosylation and S-glutathionylation from irreversible oxidation and promote the reduction of the active-site cysteinyl residue [75, 76]. For example, S-

nitrosylation of protein-tyrosine phosphatase 1B (PTP1B) Cys-215 residue protects against H_2O_2 induced oxidation demonstrating that NO effectively inhibits ROS-induced irreversible oxidation of endogenous PTP1B [77]. Src homology 2 (SH2) domain phosphatase-1 (SHP-1) is a cytoplasmic protein tyrosine phosphatase expressed in hematopoietic and vascular cells [78] that serves an inhibitory role in signaling transduction [79]. Interestingly, SHP-1 inhibition enhances VEGF-induced ROS generation in HUVEC's that could further augment ROS mediated angiogenic signaling [80]. Several other phosphatases (PTP1B, SHP2, LMW-PTP, PTEN, MAPK phosphatase) are also inhibited by redox modification [81]; however, the overall angiogenic impact of redox modulation of these phosphatases remains.

ROS and Protein Kinase C activity

PKC is a family composed of 12 isozymes divided into three classes: conventional, novel, and atypical. All PKC regulatory domains lie in the N-terminal and catalytic domains in the C-terminal. Both the regulatory and catalytic domains of PKC contain cysteine-rich regions that may be redox sensitive [82]. It has been reported that a concentration of 1 mM H_2O_2 can induce activation of PKC in rat melanoma cells [83]. However, this varies by cell type with murine macrophage and endothelial cells requiring 100-300 μM H_2O_2 to induce PKC activation [84, 85].

In bovine pulmonary artery endothelial cells (BPAEC) treatments with 1mM H_2O_2 resulted in increased activation of diacyl glycerol (DAG) and thereby PKC within 45 min [86]. It was demonstrated in bovine pulmonary microvessel endothelial cells (BMVEC), that treatments with low concentrations of H_2O_2 (0-0.5 mM) resulted in a concentration-dependent increase in PKC levels and endothelial permeability [87]. This effect of H_2O_2 has been attributed to a rise in PLC mediated intracellular Ca^{2+} release followed by activation of the p44/42 MAP kinase during normal, ischemic and ischemic/reperfusion conditions [88, 89]. Depending on cell types and the conditions prevailing (lower or higher levels), ROS has an important role in regulating PKC mediated vascular growth and remodeling.

The cellular importance of H_2O_2 mediated signal activation is probably best illustrated during changes in endothelial permeability that is considered an initial endothelial cell activation response involved in vascular growth. H_2O_2 -induced permeability effects could be aggravated under relatively high levels of NO, which may be due to singlet oxygen produced from H_2O_2 [90]. NO also activates protein kinase C α (PKC α) resulting in increased EC proliferation and migration that may further influence endothelial permeability or proliferation responses [91, 92]. Other secondarily derived oxidants, such as hydroxyl radical ($\text{OH}\bullet$) generated from decomposition of H_2O_2 coupled with significant decrease in cell glutathione (GSH) may also contribute to altered endothelial cell activation and permeability [93–95]. In addition, Caldwell and colleagues have recently shown that peroxynitrite (ONOO^-) along with H_2O_2 increases PKC activity and subsequent RhoA/Rho kinase activation that mediates increase arginase I expression in endothelial cells, which could result in L-arginine catabolism thereby limiting NO synthesis [96]. Furthermore, it is possible that H_2O_2 effects on endothelial permeability and PKC may involve alterations in tyrosine kinase activity [97]. This is confirmed through studies performed by Kevil et al

reporting the importance of tyrosine kinase/phosphatase activity in relation to increased endothelial solute permeability in bovine pulmonary endothelial cells (BPAEC) [98].

ROS and ERK1/2 activity

Extracellular signal-regulated kinases (ERK1/2) are principal signaling mediators involved in regulation of EC proliferation. Lee et al have reported that 0.5mM H₂O₂ treatment of HUVEC cells 30 minutes inhibits ERK1/2 phosphorylation but promotes p38 MAPK phosphorylation [99]. Separately, examination of isolated microvascular ECs from the heart of p47phox^{-/-} mice, Chen et al demonstrated that p47phox is a critical component of NOX induced ROS and ERK phosphorylation that is necessary for cell migration and capillary growth [100]. However, Yang et al found that 0.5 mM H₂O₂ treatment of bovine aortic endothelial cells enhances phosphorylation of ERK1/2 associated with endothelial cell apoptosis [101]. Thus, the effect of H₂O₂ on ERK1/2 activity is likely endothelial cell type specific resulting in differential cellular responses.

NO derived reactive species also significantly influence ERK1/2 activation. ONOO⁻ can indirectly promote ERK1/2 activation via stimulation of EGFR dimerization and phosphorylation of EGFR leading to activation of Ras/MAPK 1/2. Interestingly, NO itself can also promote EGFR autophosphorylation through ERK1/2 activation [102]. NO influences multiple signaling pathways through direct protein S-nitrosylation and/or stimulation of sGC dependent cGMP production that further activates Ras, protein kinase G (PKG) and downstream Ras–Raf–MEK/ERK (MAPK (mitogen-activated protein kinase) pathway resulting in increased cell proliferation and migration [91, 103, 104].

ROS and p38 MAP Kinase activity

Endothelial p38 (stress activated protein kinase-2) MAP kinase stimulation has been reported to be important for VEGF mediated stress fiber formation and cell migration, which are important for angiogenic activity [105]. Redox activation of p38 by 1 μM H₂O₂ in endothelial cells has been reported and found to be biphasic, with two activation peaks at 5 and 45-minutes, respectively. Likewise, Inhibition of p38 activity with SB203580 blocks H₂O₂-induced endothelial actin reorganization and focal adhesion assembly [106]. H₂O₂ induced endothelial cell apoptosis also involves p38 MAP kinase activation. Treatment of bovine aortic endothelial cells (BAEC) with 0.75 mM H₂O₂ for 6 hours induces endothelial cell DNA nicks and fragmentation and cleavage of caspase-3, along with concomitant phosphorylation of p38 MAP kinase. Importantly, pretreatment with the p38 MAP kinase inhibitor SB203580 significantly attenuated H₂O₂-induced caspase-3 cleavage in BAEC [107]. This oxidant regulation of p38 MAPK activity plays important roles in modulating endothelial cell architecture changes affecting migration, permeability, and survival.

ROS and transcription factor activation

ROS regulates transcription factor activation of HIF-1α, Ets-1 and NFκB that influence expression changes during angiogenesis. HIF-1α is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits that critically regulates angiogenic gene expression during hypoxia [108, 109]. HIF-1α accumulation in cells occurs under hypoxia via decreased prolyl hydroxylase activity resulting in diminished protein dehydroxylation

preventing ubiquitin mediated degradation or in non-hypoxic cells stimulated with inflammatory mediators, cytokines, reactive oxygen (ROS) and reactive nitrogen (RNS) species [110]. HIF-1 α protein can be stabilized and its activity upregulated through NO-mediated S-nitrosylation at Cys533 in the oxygen-dependent degradation domain, thus preventing its destruction [111–113]. NOX derived ROS are also involved in induction of HIF-1 α expression under normoxia and hypoxia in endothelial cells [114, 115]. ROS mediated HIF-1 expression is further enhanced by Rac1 thereby attributing the prominent role played by Rac1/Nox/ROS pathways in upregulation of HIF-1 α and VEGF expression [116]. Moreover, exogenously applied H₂O₂ (ranging from 2-250 μ M for 6 hours) to bovine aortic endothelial cells did not alter HIF-1 α protein expression; whereas, O₂^{•-} generated by xanthine oxidase (XO) and hypoxanthine (HX) dose dependently increased HIF-1 α protein levels involving JNK, p38, and PI3K/AKT pathway activity [117]. Together, these studies highlight the complex effect, of ROS on HIF-1 α expression, which contributes to endothelial cell angiogenic activity under various conditions.

Ets-1 is a transcription factor that binds to the Ets binding motif in cis-acting elements that differentially regulates expression of numerous other signaling molecules and genes. In vitro studies with BAECs show that Ets-1 mRNA levels are significantly increased after exposure to 1 μ M H₂O₂ for 2 hours [55]. Furthermore, using Ets gene deficient mice it has been reported that Ets-1 is a critical transcriptional regulator of Ang-II induced ROS formation and NOX subunit p47phox expression [118]. A recent study with proline-rich tyrosine kinase (PYK2)- apolipoprotein E (ApoE)-deficient (PYK2-KO/ApoE-KO) mice further revealed that ROS-mediated TNF α -dependent atherogenic stimulus occurs via p21Cip1/Ets-1 transcriptional activity [119]. Ets-1 also regulates the expression of genes such as proteases including matrix metalloproteinase-1 (MMP-1) and urokinase plasminogen activator (u-PA). Finally, MMP function can be directly regulated by ROS through oxidation of cysteine residues controlling enzyme activation that is involved in both physiological and pathological angiogenesis [120, 121]. Together, these studies demonstrate the relationship between ROS and Ets activation/induction for endothelial cell activation and angiogenesis activity associated with pathophysiological conditions.

NF- κ B is another critical transcription factor regulating inflammatory, apoptosis, cell proliferation and angiogenesis associated genes. Csuszar et al. demonstrated that H₂O₂ induces NF- κ B activation in endothelial cells in a concentration dependent manner [122]. Shono et al have shown that H₂O₂ (0.1 to 0.5 mM for 15 minutes) induces tubular morphogenesis of human microvascular endothelial cells by stimulating IL8 expression via NF- κ B activation [123]. Thus, redox regulation of angiogenic transcriptional pathways is closely associated with activation of upstream signaling pathways discussed above.

D. Redox regulation of vascular growth and remodeling

ROS regulation of diabetic vascular growth and complications

It is well accepted that increased ROS production during diabetes contributes to vascular and tissue damage [124, 125]. It has been reported that high glucose and advanced glycation end products increase ROS formation in VSMC [126], endothelial cells [127] and isolated arterial segments [128]. Diabetes pathogenesis also involves decreased tissue glutathione,

impaired endothelial-dependent relaxation, and increased NOX activity [129, 130]. mRNA expression of the major NOX subunits p22phox and gp91phox are elevated in vessels from diabetic animals [130], as well as p22phox, p47phox, and p67phox proteins in vessels from diabetic patients [129]. Pharmacological inhibition of NOS has also been reported to decrease $O_2^{\bullet-}$ production with NOS dysfunction being further corrected with tetrahydrobiopterin implicating NOS uncoupling and decreased nitric oxide production during diabetes [129]. Xanthine oxidase activity also accounts for significant hyperglycemia induced ROS production in diabetic tissue [131].

Peripheral angiogenic responses are significantly blunted during diabetes. There are several potential explanations for defective peripheral vascular growth during diabetes with redox imbalance playing an important role in these processes [132–134]. Postnatal vasculogenesis responses are affected by the proangiogenic phenotype of bone marrow mononuclear cells (BM-MNCs) and endothelial progenitor cells (EPCs) that are reduced in diabetic mice and patients with either type 1 or type 2 diabetes involving increased $O_2^{\bullet-}$ production, reduced NO bioavailability, and peroxynitrite formation [135–137]. Antioxidant defenses are also reduced in animal models of diabetes and contribute to diabetes-induced oxidative stress [138]. Data suggests that ROS scavenging by antioxidant treatment augments post ischemic neovascularization responses during diabetes [139]. These data reveal that ROS production during diabetes affects several different angiogenic mechanisms.

In contrast to defective peripheral angiogenic activity, increased retinal angiogenesis is observed in diabetic patients with increased $O_2^{\bullet-}$ and H_2O_2 levels detected in diabetic retinal tissue both in vitro and in vivo [140]. Moreover, antioxidants enzymes such as SOD, GSH, glutathione reductase, glutathione peroxidase and catalase are also diminished in the diabetic retina [141]. This redox imbalance activates PKC signaling pathways that in turn increase VEGF and IGF expression in the diabetic retina [140]. VEGF expression may also be elevated in diabetic retinopathy by AGE dependent increased ROS production [142]. H_2O_2 can also directly stimulate migration and proliferation in endothelial cells with concomitant VEGF-A expression and vascular smooth muscle cell proliferation [142]. These studies reveal that increased retinal angiogenesis through differential redox signaling pathways may be important for initiation of diabetic retinopathy.

Conversely, ROS also activates caspase pathways that accelerates apoptosis of diabetic retinal capillaries. Induction of NF- κ B by ROS can increase pro-inflammatory cytokine expression, NO and prostaglandin formation [143]. Proinflammatory cytokines including interleukin IL-1b, IL-6, IL-8 are increased in the vitreous fluid of diabetic patients and in the retina of diabetic rat and mice [144]. Studies have shown that IL-1b stimulates retinal capillary cell apoptosis via activation of NF- κ B and caspase 3 pathway [145]. Moreover, increased nitric oxide (NO) production via NF- κ B induction of iNOS expression can facilitate peroxynitrite (OONO $^-$) formation resulting in PARP activation and mitochondria dependent apoptosis activity [146]. Together, these events culminate in the formation of retinal acellular capillaries and development of retinopathy associated blindness.

ROS in tumor angiogenesis

Although it is understood that ROS contributes to cancer initiation and progression by oxidative modification of macromolecules such as DNA, RNA and proteins, studies also suggest that ROS promotes angiogenic signaling pathways governed by cytokines and transcription factors influencing tumor angiogenesis. Evidences from the literature demonstrate that increased redox modulation during tumorigenesis potentiates several angiogenesis events involving increased EGF, insulin, and angiopoietin-1 mediated ROS production in vascular cells regulating migration and proliferation responses [67, 147]. Additionally, elevated levels of ROS inducing DNA damage contributes to genomic instability and tumor initiation [148] involving mitogen-activated protein (MAP) kinase, nuclear factor κ B (NF- κ B), and Activator Protein 1 (AP-1) expression, which are associated with tumor angiogenesis and cancer development [149].

VEGF is a classic, well studied mediator of angiogenesis and tumor growth [150, 151]. Xia et al showed that NOX induced ROS formation regulates tumor-induced angiogenesis through HIF-1 stabilization and VEGF expression in ovarian cancer [22]. Additional evidence also indicates that mitochondrial complex III derived ROS triggers HIF-1 α activation and that inhibition of complex III blocks ROS generation preventing HIF-1 α stabilization and transcriptional activity [152]. Carcinogen induced ROS formation also increases HIF-1 α and VEGF expression via the ERK/AKT signaling pathway leading to tumor angiogenesis and progression. [153]. Finally NOX or mitochondrial complex I inhibition inhibits VEGF transcriptional activation, mRNA and protein levels reinforce that redox stress plays an important role in regulating tumor mediated angiogenesis via VEGF expression [151]. Together, these and other findings clearly demonstrate increased ROS formation positively influences tumor vascular growth.

Regulation of ROS in ischemic vascular growth

Deprivation of tissue blood flow in either an acute or chronic manner results in tissue ischemia that activates several compensatory responses influencing vascular growth and development to restore perfusion to ischemic tissue. Evidence has shown that the response to hypoxia/ischemia is strongly influenced by increased ROS production leading to increased VEGF and VEGF receptor expression that is also linked to a concomitant increase in ROS production during hypoxia as mentioned previously [70, 154]. Exogenous application of ROS promotes endothelial responses that mimic angiogenesis such as increased cell proliferation, migration, and tube formation. These responses involve HIF-1 α activation due to protein stabilization and increased ROS flux during hypoxia that is absent in anoxia [155, 156]. Low levels of ROS, such as $O_2^{\bullet-}$ and H_2O_2 can act as intracellular signaling molecules to regulate cell growth, differentiation, and angiogenesis invoking various kinase cascades discussed above [13, 45, 70]. Moreover, moderate elevation of ROS production induces VEGF expression and promotes endothelial differentiation from embryonic stem cells [157, 158]. Studies have also shown that pretreatment of bone marrow cells with H_2O_2 enhanced their survival rate, expression of VEGF and Flk-1, and endothelial differentiation and function in vitro and in vivo [46, 55, 154, 159]. As might be expected, increased neovascularization in response to ischemia or VEGF is inhibited in NOX2^{-/-} mice or wild-type mice treated with antioxidant ebselen or NOX blocking agents apocynin

or gp91ds-tat [45, 160] consistent with the fact that NOX2 expression is increased during experimental ischemia hind limb. It has also recently been reported that augmentation of endothelial NOX4 expression promotes angiogenesis and recovery of blood flow in the mouse ischemic hind limb through enhanced eNOS activation [69]. Lastly, deficiency of antioxidant response factors significantly influence ischemia-induced neovascularization such that Nrf2^{-/-} deficient mice display increased oxidative stress during ischemia with a surprising concomitant increase in endothelial progenitor cell contribution to neovascularization [161]. Thus, increased ROS formation is clearly involved in signaling responses important for ischemic angiogenesis.

However, over or sustained production of ROS may result in cellular toxicity and has been implicated in impaired angiogenesis responses in different models such as diabetes and atherosclerotic vessel disease such that NOX2 deficiency augments ischemic angiogenesis responses during these pathologies [162]. Correction of the impaired angiogenesis response was associated with reduced ROS formation, restored activation of the VEGF/NO angiogenic signaling pathway, and improved function of EPCs [163]. Therefore, oxidative stress during ischemic angiogenesis may act in a duplicitous manner by either activating or inactivating angiogenic signaling in healthy or diseased tissue states, respectively.

E. Molecular redox regulators of angiogenic activity

Thioredoxins

Thioredoxin proteins (Trx-1 and 2) play critical roles in vascular health and physiology via redox regulation of endothelial signaling (e.g. ASK-1) and cell stress responses (e.g. hypoxia) [164]. Thus, it is logical that Trx-1 and 2 could serve important roles in redox regulation of vascular growth and remodeling responses. Welsh et al has shown that Trx-1 augments HIF-1 α expression and activity during hypoxia requiring functional redox activity as Cys mutations blunted this regulation [165]. Importantly, Trx-1 modulation of HIF-1 α activity influences VEGF expression controlling tumor angiogenesis activity [165]. Similarly, Takagi et al recently reported that Trx-1 dependent HIF-1 α activity is also associated with cerebral arteriovenous malformations further implicating the importance of Trx-1/HIF-1 α activation [166]. These studies indicate that Trx-1 may alter key signaling events involved in pathological angiogenesis responses typically seen during cancer.

However, thioredoxin protein function may also augment angiogenic activity in a beneficial manner as previously reported in experimental models of permanent tissue ischemia. A report from Samuel et al showed that adenoviral Trx-1 overexpression protects against diabetic myocardial ischemia due to permanent left anterior descending (LAD) artery ligation [167]. AdTrx-1 overexpression was associated with reduction of oxidative stress and preservation of myocardial vascular density associated with p38 MAPK and HO-1 activation, and VEGF expression. Similarly, transgenic overexpression of Trx-1 also protects against myocardial ischemic injury associated with decreased oxidative stress and apoptosis along with increased vascular density of the myocardium [168]. These observations were further associated with increased phosphorylation of AKT, eNOS, and GSK3 β concomitant with increased β -catenin and HIF-1 α activity. Endothelial cell specific transgenic overexpression of mitochondrial Trx-2 also augments ischemic angiogenesis in

the unilateral permanent femoral artery ligation (FAL) model [169]. Dai and colleagues showed that endothelial cell specific Trx-2 transgenic mice showed enhanced ischemic vascular remodeling responses (angiogenesis and arteriogenesis) that was associated with increased NO bioavailability and decreased oxidative stress and phosphorylation of ASK-1. Lastly, Dunn et al has previously mentioned that inhibition of TXNIP up-regulation during streptozotocin induced diabetes augments the typical impaired angiogenic response in the diabetic FAL model although the precise details and mechanisms of this response are not yet clear [170]. These observations together with those from tumor angiogenesis studies demonstrate that members of the thioredoxin protein family clearly modulate different aspects of ischemic vascular growth and remodeling; however, the role and importance of these proteins during pathological versus physiological angiogenesis activity requires further definition and understanding.

Glutathione generation and metabolism

Glutathione (GSH) and its metabolism serve as a primary and dominant redox buffering system within cells and tissues. Regulation of GSH metabolism critically controls cell survival and growth including numerous signaling responses involving protein posttranslational modifications (i.e. glutathionylation) and counterbalance against endogenous reactive oxygen species generation [171, 172]. Given the critical nature of these cellular and biochemical functions and the importance of redox balance for controlling vascular growth, relatively few studies compared to classical growth factor studies have closely evaluated the role of GSH and its metabolism on redox regulation of vascular growth and development.

The importance of GSH metabolism for endothelial cell growth has been reported by Mallery et al who examined bioenergetic and glutathione profiles during proliferation and differentiation in human endothelial cells from the retinal microvasculature (HMECs) [173]. This study found that total GSH levels were most abundant in proliferating HMECs and that as the endothelial monolayer progressed through predifferentiation to full differentiation GSH levels significantly decreased. This observation is consistent with the numerous studies discussed above revealing that growth factor stimulation of signaling pathways invokes ROS production that is associated with growth and proliferation thus increased GSH would be necessary for proper regulation of numerous intracellular metabolic responses. Consistent with this hypothesis studies have shown that altering GSH levels significantly impacts tumor angiogenic responses. Albini et al reported that oral administration of N-acetyl cysteine (NAC) significantly blunted established Kaposi's sarcoma growth in animal models that was associated with decreased tumor vascularity, MMP activity and VEGF expression [174]. Schwartz et al also found that exogenous GSH administration blunted DMBA mediated oral carcinogenesis involving decreased angiogenesis and p53 expression [175]. These studies are consistent with work from Sihvo et al that reported an inverse relationship between GSH levels and gastroesophageal cancer such that GSH levels were higher in normal gastroesophageal biopsies and significantly lower in biopsies of Barrett's esophagus and esophageal cancer [176]. Together, these reports implicate a role for GSH metabolism regulating vascular growth yet specific cellular responses and molecular mechanisms were unknown.

Recent work from ours and other labs has begun to address possible molecular mechanisms involved in GSH regulation of angiogenesis. In a series of studies aimed at understanding endothelial cell adhesion molecule (ICAM-1) function using genetic and molecular approaches, we discovered that genetic mutation or antibody crosslinking and signaling of ICAM-1 significantly increased endothelial cell total GSH levels along with an increase in the reduced to oxidized ratio (GSH:GSSG) [177, 178]. Importantly, ICAM-1 mutant endothelial cells were found to be resistant to VEGF mediated angiogenic activation that was mediated by elevated GSH levels [179]. We further found that the cytoplasmic tail of ICAM-1 serves as a critical regulator of endothelial NOX4 activity that initially increases ROS formation that subsequently activates the rate limiting GSH synthesis enzyme, glutamate cysteine ligase (GCL) [180]. These findings are consistent with a report by Tajima et al that further reported increased intracellular GSH decreases HIF-1 α activity, whereas increased intracellular GSSG increased HIF-1 α activity [181]. Interestingly, Galasso et al reported that genetic deficiency of glutathione peroxidase-1 (GPx-1) significantly impairs ischemic revascularization responses that are associated with defective EPC mobilization to ischemic tissues [182]. These results indicate the existence of a 'GSH threshold' that critically modulates endothelial cell angiogenic activation and that alteration of GSH metabolism differentially affects various cell types involved in vascular growth and repair. While these reports are informative, additional studies are clearly needed to better understand specific mechanisms of increased GSH dependent inhibition of angiogenic activity.

Peroxiredoxins

The peroxiredoxin family of proteins (Prx1-6) provides intracellular regulation of intracellular peroxide functions through their peroxidase activity via oxidation of Cys-SH to Cys-SOH that can be reduced by sulfiredoxin and GSH [183]. In this manner, peroxiredoxins critically control many intracellular signaling pathways involved in cell growth and survival. Studies have examined the function of Prx-1 and Prx-2 on endothelial and vascular function under various conditions. Both Prx-1 and Prx-2 have been implicated in regulating endothelial cell activation during atherosclerosis such that genetic deficiency of either gene augments atherogenesis in Apo-E deficient mice [184, 185]. Moreover, exposure of endothelial cells to laminar shear stress up-regulates Prx-1 highlighting its importance as a regulator of endothelial cell redox potential [186]. Likewise, Prx-1 and Prx-2 have been implicated in tumor angiogenesis. Prx-1 has been reported to modulate prostate tumor growth via a TLR4 regulation of tumor angiogenesis [187]. In this study, the authors found that inhibition of Prx-1 reduced VEGF expression within ectopic prostate cancer in rodents that was associated with reduced tumor growth and progression. Likewise, Lee et al reported that Prx-2 expression was elevated in endothelium of benign vascular tumors but that it was significantly reduced in endothelial cells of malignant tumors of Kaposi's sarcoma and angiosarcoma [188]. This is consistent with the possibility that diminished Prx-2 may facilitate angiogenic activity which has been recently been elucidated. Kang et al recently reported that Prx-2 critically regulates redox inactivation of VEGFR2 such that genetic deficiency of Prx-2 prevents VEGF mediated VEGFR2 activation and angiogenesis as well as tumor angiogenesis [189]. Results from this study revealed that Prx-2 serves as a critical antioxidant defense to prevent VEGFR2 thiol oxidation and

disulfide formation. While there are currently few studies specifically addressing Prx protein function for vascular growth and remodeling, it is clear that their roles will be complicated such that they may positively or negatively regulate angiogenesis depending on the prevailing redox status of the endothelium. These studies highlight the dynamic and complex nature of vascular and endothelial cell redox biology that should be given careful consideration for future studies.

F. Conclusions and future directions

It is now abundantly clear that redox regulation mechanisms involving ROS production and metabolism play critical roles in modulating vascular growth and remodeling under both physiological and pathological conditions. Disease processes characterized by either increased or defective vascular growth also manifest dysfunctional antioxidant regulatory pathways influencing ROS/RNS bioavailability. However, the roles and importance of antioxidant regulation of vascular growth remains poorly understood by comparison. It is critical that future studies investigating redox regulation of vascular growth and remodeling address this topic as it is integral for cell survival in ROS rich environments, such that decreased endothelial cell antioxidant capacity likely contributes to growth and apoptosis responses in a time and/or concentration dependent manner. Importantly, determining specific thresholds of antioxidant activity versus ROS production is crucial for further understanding the pathophysiological etiology of vascular growth and remodeling.

It is also increasingly evident that many of the discussed redox regulation responses are different among various vascular cell types with the possibility that endothelial cells from specific organ beds respond to redox dependent angiogenesis in distinctly unique manners. Future studies addressing vascular cell specificity responses will be key in discerning why different organs manifest divergent angiogenic responses as can be seen during diabetes. Finally, specific detection and quantitative analysis of various different ROS and RNS species have significantly evolved at the analytical level; yet, few of these refined techniques and approaches have been employed to precisely determine the amount and redox chemical species necessary for both physiological and pathological angiogenesis. Moving forward, it is important that future studies on redox regulation of angiogenesis and vascular remodeling keep these issues in mind to elucidate greater understanding and insight of redox mediated vascular growth mechanisms that are necessary for development of novel therapeutic approaches targeting the vasculature in health and disease.

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Abbreviations

ASK-1	Apoptosis signal-regulating kinase 1
BAEC	Bovine aortic endothelial cells

bFGF	Basic fibroblast growth factor
BMEC	Bovine pulmonary microvessel endothelial cells
BM-MNCs	Bone marrow mononuclear cells
BPAEC	Bovine pulmonary artery endothelial cells
COX 2	Cyclooxygenase 2
EGFR	Epidermal growth factor receptor
eNOS	Endothelial nitric oxide synthase
EPCs	Endothelial progenitor cells
ERK	Extracellular signal-regulated kinases
GCL	Glutamate cysteine ligase
GPx-1	Glutathione peroxidase-1
GSH	Glutathione
GSK3β	Glycogen synthase kinase 3 beta
GTPase	Guanosine-5'-triphosphatase
HIF-1α	Hypoxia inducible factor
HMECs	Human endothelial cells from the retinal microvasculature
HUVEC	Human umbilical vein endothelial cells
ICAM	Intercellular Adhesion Molecule
iNOS	Inducible nitric oxide synthase
JNK	c-Jun N-terminal kinases
L-NAME	N-nitro-L-arginine methylester
MAP kinase	Mitogen-activated protein (MAP) kinases
MMP	Matrix metalloproteinase
NAC	N-acetyl cysteine
nNOS	Neuronal nitric oxide synthase
NOX	Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet derived growth factor
PGF2	Prostaglandin F2
PKC	Protein kinase C
PLC	Phospholipase C
PPARα	Peroxisome proliferator-activated receptor α

Prx	Peroxiredoxin
PTP	Protein tyrosine phosphatases
PTEN	Phosphatase and tensin homolog
PTP1B	Protein-tyrosine phosphatase 1B
PYK2	Proline-rich tyrosine kinase
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
SDF-1	Stromal cell-derived factor-1
SOD	Superoxide dismutase
Trx	Thioredoxin
TXNIP	Thioredoxin-interacting protein
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
XDH	Xanthine dehydrogenase
XO	Xanthine oxidase

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Figure 1. ROS dependent modulation of vascular growth responses for physiological and pathological conditions

The left side of the figure highlights physiological angiogenesis examples that involve low regulated levels of ROS production. The right side of the figure lists pathological angiogenesis situations that involve abundant or prolonged ROS generation contributing to various disease processes.

Figure 2. Major enzymatic and intracellular sources of ROS production

Membrane associated NADPH oxidase (NOX) complex assembly is a major source of $O_2^{\bullet-}$ production; however, the NOX4 isoform may also constitutively produce H_2O_2 . eNOS uncoupling produces $O_2^{\bullet-}$ due low bioavailability of cofactors NADPH, BH_4 and/or L-arginine substrate that can then react with NO to form peroxynitrite. XO produces both superoxide and H_2O_2 with $O_2^{\bullet-}$ production occurring under higher O_2 tension (10-21% O_2) and H_2O_2 generation at lower O_2 tension (1% O_2). $O_2^{\bullet-}$ is also dismutated to H_2O_2 by Cu and Zn dependent superoxide dismutase (CuZnSOD) within the cytosol. Mitochondria also produce $O_2^{\bullet-}$ and H_2O_2 due to respiratory chain dysfunction (RCD) that regulated by Mn dependent superoxide dismutase (MnSOD).

Figure 3. Prominent angiogenic signaling pathways regulated by ROS

ROS production from all sources; NOX isoforms, uncoupled eNOS, XO and mitochondria directly influence numerous signaling pathways (e.g. PKC, RTK, p38 MAPK, and ERK1/2) involving both inhibition of regulatory phosphatase activity or activation of kinase activity. Subsequent redox dependent signaling stimulates activation of various transcription factors including HIF-1, NF-kB, Ets and others followed by up-regulation of angiogenic molecules

such as VEGF, MMP and uPA that stimulate endothelial cell proliferation and migration thus increasing angiogenesis activity.