Published in final edited form as:
Cancer Lett. 2008 July 18; 266(1): 37–52. doi:10.1016/j.canlet.2008.02.044.

Reactive Oxygen Species and Angiogenesis: NADPH Oxidase as Target for Cancer Therapy

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Abstract

Angiogenesis is essential for tumor growth, metastasis, arteriosclerosis as well as embryonic development and wound healing. Its process is dependent on cell proliferation, migration and capillary tube formation in endothelia cells (ECs). High levels of reactive oxygen species (ROS) such as superoxide and H_2O_2 are observed in various cancer cells. Accumulating evidence suggests that ROS function as signaling molecules to mediate various growth-related responses including angiogenesis. ROS-dependent angiogenesis can be regulated by endogenous antioxidant enzymes such as SOD and thioredoxin. Vascular endothelial growth factor (VEGF), one of the major angiogenesis factor, is induced in growing tumors and stimulates EC proliferation and migration primarily through the VEGF receptor type2 (VEGFR2, Flk1/KDR). Major source of ROS in ECs is a NADPH oxidase which consists of Nox1, Nox2, Nox4, Nox5, $p22^{phox}$, $p47^{phox}$ and the small G protein Rac1. NADPH oxidase is activated by various growth factors including VEGF and angiopoietin-1 as well as hypoxia and ischemia, and ROS derived from this oxidase are involved in VEGFR2 autophosphorylation, and diverse redox signaling pathways leading to induction of transcription factors and genes involved in angiogenesis. Dietary antioxidants appear to be effective for treatment of tumor angiogenesis. The aim of this review is to provide an overview of the recent progress on role of ROS derived from NADPH oxidase and redox signaling events involved in angiogenesis. Understanding these mechanisms may provide insight into the NADPH oxidase and redox signaling components as potential therapeutic targets for tumor angiogenesis.

Introduction

Angiogenesis, the process of new blood vessel formation from the pre-existing vessels, plays an important role in tumor growth, metastasis, embryonic development, wound healing, and arteriosclerosis [1]. It is required for tumors to grow beyond a few millimeters in diameter, because successful growth and metastasis of tumors requires the establishment of an efficient blood supply [2]. Progression to a growing tumor is characterized by induction of proangiogenic factors such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), and VEGF receptors (VEGFR) in the growing endothelial cells (ECs)(Figure 1). VEGF is one

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of the most important angiogenesis growth factor [3] and stimulates permeability, proliferation, migration and tube formation of ECs primarily through the VEGF receptor type2 (VEGR2, KDR/Flk1) [4](Figure 1). VEGF plays a critical role in tumor angiogenesis not only through its effect on EC but also through mobilization of bone marrow-derived endothelial progenitor cells [5]. Many stimuli including hypoxia, growth factors, cytokines and oxidative stress can increase VEGF expression in tumor cells, which is correlated with increased microvessel counts and poor prognosis in many human cancers (Figure 1). The conversion to the angiogenic phenotype in previously dormant tumors is known as the "angiogenic switch". Thus, anti-angiogenic therapy is essential strategy for the treatment of tumor [2, 6], which has been focused on targeting VEGF and VEGFR2 using specific antibodies and tyrosine kinase inhibitors (Figure 1).

Reactive oxygen species (ROS) such as such as superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) are found in a large number of tumors [7]. ROS are conventionally thought as cytotoxic and mutagenic, and in high levels they induce cell death, apoptosis and senescence. In contrast, ROS at low levels function as signaling molecules to mediate cell growth, migration, differentiation and gene expression. Of note, ROS play an important role in angiogenesis [8, 9]. ROS are produced in response to hypoxia, ischemia, angiogenic growth factors such as VEGF and angiopoietin-1, thereby stimulating EC proliferation and migration [10–12]. ROS in tumor cells also contribute to angiogenesis and mitogenesis [13–15]. Anti-tumor activity of conventional chemotherapeutic agents is enhanced by antioxidants treatment [16]. Tumor cells may be inherently more resistant to oxidative stress than normal cells, or oxidative stress may provide a selective advantage in tumor growth. ROS-generating enzymes such as NADPH oxidase (Nox) has been shown to contribute to activation of redox signaling pathways leading to angiogenic responses in ECs as well as postnatal angiogenesis *in vivo* [11, 17–25]. The aim of this review is to summarize the recent progress and information on ROS and NADPH oxidase as potential therapeutic targets for treatment of tumor angiogenesis (Figure 1).

1. Generation and metabolism of ROS

Oxygen is fundamental to cellular respiration and cells have evolved several enzyme systems that use this ubiquitous substrate as an acceptor of electron transfer. The reduction of oxygen by one electron increases the formation of O_2^{-}, which can be either dismutated to H_2O_2 spontaneously or in a reaction catalyzed by superoxide dismutase (SOD). Further reactions lead to the formation of hydroxyl radicals ($^{\bullet}OH$), especially in the presence of metal ions through the Fenton or Haber-Weiss reactions [26, 27]. O_2^{-} reacts with nitric oxide ($NO^{\bullet}$) at a near diffusion-limited rate to form peroxynitrite ($ONOO^{-}$), which is a potent oxidant. $NO^{\bullet}$ is protective and thus loss of $NO^{\bullet}$ bioavailability via these reactions contributes to various pathophysiologies. Mammalian peroxidases such as myeloperoxidase (MPO) are activated by H_2O_2 to form a highly reactive radical that can oxidize $NO^{\bullet}$ to NO_2^{-} and react with NO_2^{-} to form $NO_2^{\bullet}$. $NO_2^{\bullet}$ can, in turn, participate in nitrating events, such as the formation of nitrotyrosines. Under homeostatic conditions, antioxidant defences are critical to modulate the steady state balance.

2. Role of ROS in Angiogenesis

ROS such as such as $O_2^{\bullet -}$ and H_2O_2 are increased in a large number of cancer cells [7]. ROS act as key mediators of the cellular signaling induced by the ligation of the cell surface receptors as well as by many classes of environmental agents [8, 28–32]. Cell stimulation by such agents has been shown to increase the cellular ROS levels, which regulate various cellular functions. Exogenous ROS stimulate induction of VEGF by various cell types, and promote cell proliferation and migration [10, 12, 13, 33], cytoskeletal reorganization [34] and tubular morphogenesis [35] in ECs. Hypoxia/reoxygenation and adhesion of activated

polymorphonuclear leukocytes to ECs causes ROS production, which results in capillary tube formation [36, 37]. Angiogenesis growth factors such as VEGF and angiopoietin-1 (Ang1) induce EC migration and/or proliferation through an increase in ROS [11, 21, 23, 25]. Leptin, a circulating adipocytokine, is also defined as angiogenesis factor [38] and upregulates VEGF mRNA and stimulates cell proliferation through an increase in ROS in ECs [39]. Pigment epithelium-derived factor (PEDF), a natural inhibitor of angiogenesis [40], blocks leptin-induced ROS production in ECs [39], suggesting that it may function as an endogenous antioxidant factor. Thus, many of angiogenenic-related responses are mediated through ROS in ECs.

In vivo, ROS are also involved in tumor angiogenesis as well as various physiological and pathological angiogenesis. ROS play an important role in neovascularization during tumor growth [41], and thiol antioxidant, N-acetylcysteine (NAC) attenuates EC invasion and angiogenesis in a tumor model *in vivo* [42]. PEDF which has antioxidant properties inhibits angiogenesis and melanoma growth [43]. ROS are increased during the reperfusion of the ischemic retina, which upregulates VEGF mRNA [44]. Short periods of ischemia/reperfusion or preconditioning induce an increase in ROS, thereby stimulating myocardial angiogenesis or collateral development [45–47]. Moreover, ROS are involved in physiological repair processes such as ischemia-induced angiogenesis and wound healing *in vivo* [22, 48]. The ROS inhibitors [49] or antioxidants such as pyrrolidine dithiocarbamate [50] inhibit neovascularization in the mouse model of angiogenesis. Recently, Kim et al. [51] demonstrated that Ang1-induced angiogenesis is enhanced in catalase$^{-/-}$ mice as compared to catalase$^{+/+}$ mice, suggesting that Ang1-induced H_2O_2 plays an essential roles in angiogenesis *in vivo*. In pathophysiological state, strong correlation between ROS production, neovascularization and VEGF expression has been reported in eyes of diabetics [52–54] and balloon injured arteries [55]. Anti-angiogenic therapy reduces plaque growth and intimal neovascularization in atherosclerosis [56]. Antioxidants vitamins C and E reduce VEGF and VEGFR-2 expression in apolipoprotein-E deficient mice [57]. These reports indicate that ROS play an important role in postnatal neovascularization in physiological and pathophysiological states.

3. Role of NADPH oxidase in Angiogenesis

ROS are generated from a number of sources including the mitochondrial electron transport system, xanthine oxidase, the cytochrome p450, the NADPH oxidase, uncoupled NO synthase (NOS) and MPO. The mitochondria and the Nox family of NADPH oxidase have emerged as major sources of ROS induction [31, 58, 59]. Before the recent discovery of the NADPH oxidase family as a major source of ROS generation [18], the main intracellular ROS were known to be derived from mitochondria. Mitochondrial DNA mutations are responsible for aberrant ROS production, leading to development of neoplastic lesions and progression [60]. It is unclear whether mitochondria-dependent ROS generation are directly associated with tumor angiogenesis.

One of the major sources of ROS in ECs is NADPH oxidase, also known as Nox enzymes. The prototypical Nox is the phagocyte NADPH oxidase that is normally quiescent but generates a large amount of $O_2^{\bullet-}$ upon activation during phagocytosis via the one electron-reduction of oxygen by NADPH, which participate in host defense by killing invading microbes [59, 61]. This phagocytic type of NADPH oxidase consists of membrane-bound subunits, gp91^{phox} and p22^{phox} which form the flavocytochrome b_{558} complex, together with the cyctosolic subunits p40^{phox}, p47^{phox}, and p67^{phox} as well as small GTPase, Rac. Superoxide production is induced by assembly of the cytosolic and membrane-bound subunits, which is mediated through the phosphorylation of p47^{phox} [62]. The neutrophil NADPH oxidase releases large amounts of $O_2^{\bullet-}$ in bursts, whereas the non-phagocytic NADPH oxidase(s) continuously produce low levels of $O_2^{\bullet-}$ intracellularly in basal state, yet it can be further stimulated acutely by various agonists and growth factors [31, 63]. Several human homologs of gp91^{phox} (also termed as Nox2)

have been identified in non-phagocytic cells. The first described homolog is Nox1 which was cloned from the colon epithelial cells and involved in mitogenic activity. [17]. Subsequently, additional homologs including Nox3, Nox4, Nox5, and the Dual oxidases (Duox1, and Duox2) were cloned [59, 64–66] (Figure 2). Of note, each is encoded by different genes [59, 63]. Recently, isoforms of $p47^{phox}$ and $p67^{phox}$ were discovered. These were termed NoxO1 (for Nox organizer 1) and NoxA1 (for Nox activator 1), which substitute for $p47^{phox}$ and $p67^{phox}$ respectively [67, 68]. An important difference between $p47^{phox}$ and NoxO1 is that the latter lacks the $p47^{phox}$ domain that is regulated by phosphorylation; therefore NoxO1 may influence oxidase activity quite differently from $p47^{phox}$.

Nox isozymes have been shown to increase in association with ROS production and tumorgenicity in various cancer cells. Nox1 is highly expressed in human colon cancers and prostate cancers [69]. Overxpression of Nox1 in NIH3T3 fibroblasts induced malignant transformation, rendering them slightly tumorigenic in athymic mice [17]. NIH 3T3 cells that stably express Nox1 exhibit cell growth, and coexpression of catalase along with Nox1 reverses the growth phenotype, suggesting that one of the signaling species generated by Nox1 is H_2O_2 [18]. Arbiser et al. [19] demonstrated that Nox1-induced H_2O_2 increases VEGF and VEGF receptor expression and MMP activity, markers of the angiogenic switch, thereby promoting vascularization and rapid expansion of the tumors. In colon epithelial cells, Nox1 oxidase activity requires NoxO1 and NoxA1 instead of $p47^{phox}$ and $p67^{phox}$. In addition to Nox1, expression of Nox4 and Nox5 has been shown to be increased in melanoma cells [20] and prostate cancer cells [70], respectively. Of note, there is cross-talk between mitochondria and O_2^- generating NADPH oxidase in breast and ovarian tumors in that mitochondria controls Nox1 redox signaling and the loss of control of this signaling contributes to tumorigenesis [71].

Various Nox enzymes, Nox1, Nox2, Nox4 and Nox5 as well as cytosolic regulatory subunits $p47^{phox}$, $p67^{phox}$ and Rac1 have been shown to be involved in ROS production in ECs (Figure 2), a major site of angiogenesis as well as *in vivo* model of angiogenesis, as listed below. To our knowledge, role of Nox3, Duox, NoxO1 and NoxA1 in ECs/cancer cells has not been demonstrated.

Nox1

Nox1 is upregulated by oscillatory shear stress, mediating ROS-dependent leukocyte adhesion to ECs [72]. Nox1 stimulates branching morphogenesis in sinusoidal ECs [73]. Given that leukocyte adhesion to ECs causes disruption of cell-cell junction which is required for initiating EC migration and proliferation, and that morphogenesis is a critical component of angiogenic process, it is likely that Nox1 may be involved in angiogenesis in ECs. Of note, Petry et al. [74] showed that Nox1 depletion has no effect on ROS levels and proliferation in basal EC.

Nox2

Nox2 is a critical component of ROS-generating NADPH oxidase activated by various stimulants and agonists in ECs [11, 75–78]. VEGF and Ang1 stimulate Nox2-based NADPH oxidase in EC, which is involved in angiogenesis in ECs [11, 25]. We have shown that Nox2 binds to actin and IQGAP1, an actin- and Rac1-binding scaffold protein, at the leading edge in migrating ECs [79]. IQGAP1 seems to tether Nox2 to actin cytoskeleton at the leading edge to direct ROS production and EC migration, which may contribute to angiogenesis. Consistent with this, Petry et al. [74] reported that Nox2 is co-localized not only with endoplasmic reticulum (ER) marker calreticulin but also with F-actin at the plasma membrane. Li et al. [80] showed that IL-1b stimulation of MCF-7 epithelial cells causes Nox2 internalization into endosomes in a Rac1-dependent manner together with the IL receptor. H_2O_2 generation within the endosomes plays a critical role in subsequent activation of an IκB (inhibitory κB) kinase complex and NF-κB. Neovascularization in response to ischemia or VEGF is inhibited in

$Nox2^{-/-}$ mice and in wild-type mice treated with antioxidant ebselen or NADPH oxidase inhibitor apocynin or gp91ds-tat [11, 22, 24]. Furthermore, Nox 2 expression is increased in association with ROS production in mice ischemia hindlimb and retinopathy model [22, 24]. Khatri et al. [81] have shown that vascular NADPH oxidase-derived ROS promotes VEGF expression and neovascularization of experimental atheroma in transgenic mice overexpressing $p22^{phox}$, a binding partner of Nox.

Nox4

Nox4 is expressed more abundantly compared to other Nox proteins in ECs, and seems to be involved in basal $O_2^{\bullet-}$ production [82]. Kuroda et al. [83] demonstrated that Nox4 preferentially localizes to the nucleus in human ECs, which is involved in basal- and PMA-stimulated NADPH oxidase activity in nuclear fraction as well as oxidative stress responsive gene expression. Thus it is temping to speculate that Nox4 might act as a sensor for nuclear redox [65], which may contribute to gene expression linked to cell growth, differentiation, senescence or apoptosis. Nox4 is also localized at endoplasmic reticulum where it interacts with $p22^{phox}$, which contributes to basal ROS production and proliferation in ECs [74]. Most recently, Datla et al. [84] reported that Nox4 siRNA inhibits VEGF-induced EC migration and proliferation, while overexpression of dominant negative Nox4 blocks PDGF receptor autophosphorylation. It should be noted that this dominant negative Nox4 also blocks Nox2 function. Role of endogenous Nox4 in VEGFR2 autophosphorylation and downstream signaling remains unclear. Vallet et al. [85] reported that Nox4 expression is upregulated in new capillaries in brain ischemia-induced angiogenesis of mice.

Nox5

NOX5 contains four EF-hand domains in its N terminus, is FAD and NADPH dependent as other Nox1-Nox4 but $p22^{phox}$ independent [86] and is activated by calcium [87]. NOX5 is expressed in lymphoid cells and the testis [87] as well as in prostate cancer cells [70]. Most recently, BelAiba et al. [88] reported that Nox5β, Nox5δ as well as a short variant lacking calcium-binding domains, Nox5S are expressed in human microvascular ECs. They also showed that Nox5 is mainly localized at ER as other Nox enzymes, and that overexpression of Nox5 stimulates ROS production, proliferation and formation of capillary-like structures whereas depletion of Nox5 by siRNA prevents these responses by thrombin in ECs. Whether Nox5 is involved in VEGF or Ang-1-induced ROS production and angiogenesis remains unknown. Of note, overexpression of $p22^{phox}$ interacts with Nox5 but it is not essential for Nox5-mediated ROS production. Nox5 is present in most mammals but is absent in rodents where its function may have been taken over by another Nox or Duox. These suggest that Nox5 variants may play a role in controlling ROS-dependent process involved in angiogenesis.

Rac1

Rac1 is involved in VEGF- and Ang-1-induced increase in ROS production which is involved in EC migration, a critical angiogenesis process [11, 25]. Overexpession of active form of Rac1 induces loss of cell-cell adhesion [89] and cytoskeletal reorganization [90] through increase of H_2O_2, which are required for EC migration. Rac1 activity is highest at the leading edge in wound-induced migrating cells [91] and endogenous H_2O_2 accumulates at the membrane ruffles in actively migrating ECs [79, 92]. Thus, Rac1 seems to play an important role in angiogenesis in ECs. Of note, HMG-CoA reductase inhibitor, statins which inhibit Rac1 activity [93] can block angiogenesis *in vivo* [94].

$p47^{phox}$

In ECs, $p47^{phox}$ phosphorylation is involved in oxidase activation in response to angiotensin II, TNFα, VEGF and oscillatory shear stress, and these agonist-induced O_2^- production is

inhibited in ECs isolated from $p47^{phox-/-}$ mice [95–97]. Protein kinase C isoforms are the major kinases responsible for $p47^{phox}$ phosphorylation although other kinases such as Akt, p38 MAP kinase and PAK (p21-activated kinase) also play a role depending on the stimulus [59, 98, 99]. Gu et al. [100] reported that $p47^{phox}$ localizes to the cytoskeletal elements and plays a role in TNFα-induced c-terminal Jun kinase activation in ECV304 cells. After agonist stimulation or during directed cell migration, $p47^{phox}$ translocates from the perinucleus to the membrane ruffles through binding to the WAVE1 and to the leading edge of focal complexes through binding to the adaptor TRAF4 and Hic-5, a focal contact scaffold, in ECs [101–103]. Thus, $p47^{phox}$ is a functional component of NADPH oxidase associated with actin cytoskeleton in ECs, implicating its role in angiogenesis. Recently, Chen et al. [104] reported that Ang1-stimulated ROS, Akt and ERK phosphorylation, cell migration and capillary growth from aortic ring are inhibited in heart microvascular ECs from $p47^{phox-/-}$ mice. Thus, $p47^{phox}$ is a critical component of NADPH oxidase involved in angiogenesis in EC and isolated vessels. Definitive role of $p47^{phox}$ in postnatal angiogenesis including tumor angiogenesis *in vivo* will require further investigation using $p47^{phox-/-}$ mice.

4. Role of SOD in Angiogenesis

The reduction of oxygen by one electron increases the formation of O_2^-, which can be either dismutated to H_2O_2 spontaneously or in a reaction catalyzed by SOD. Overexpression of extracellular SOD (ecSOD) inhibits tumor vascularization in mice [105], suggesting that antioxidant ecSOD treatment is useful therapeutic strategy for angiogenesis-dependent diseases. Using ecSOD deficient mice, we recently demonstrated that endogenous ecSOD is required for reparative neovascularization in response to ischemic injury by protecting ischemic tissues and bone marrow from overproduction of O_2^- [48]. This study suggests that optimal low level of ROS is necessary but overproduction of ROS is inhibitory for postnatal angiogenesis in vivo. Furthermore, cytosolic Cu/ZnSOD overexpressing transgenic mice show enhancement of FGF-induced angiogenesis and tumor development [106], and gene transfer of Cu/ZnSOD in NIH3T3 fibroblasts enhances VEGF synthesis through an increase in H_2O_2 [107]. Connor et al. [108] have reported that MnSOD promotes mitochondrial H_2O_2 production, thereby stimulating EC sprouting and neovascularization in the CAM assay. VEGF-induced ROS produced via activation of Rac1 upregulate MnSOD expression in ECs [109], which could represent a feed-forward mechanism by which ROS-triggered H_2O_2 plays an important role in angiogenesis. Thus, SOD may serve as a H_2O_2-generating, pro-angiogenic enzyme, rather than anti-angiogenic, antioxidant enzyme, in some settings [107].

5. Role of Thioredoxin in Angiogenesis

Thioredoxins (Trx) are a family of small redox active proteins that undergo reversible oxidation/reduction and play an important role to maintain the redox state of cells. Trx serves as a cofactor in many Trx-catalyzed reductions in a manner similar to glutathione in thioltransferase reactions to control the reduced intracellular redox environment, cellular growth, and defense against oxidative stress in mammalian cells including cancer cells [110]. Trx-1 is involved in many of the hallmarks of cancer including increased proliferation, resistance to cell death and increased angiogenesis [111]. Trx-1 is a validated cancer drug target associated with aggressive tumor growth, resistance to standard therapy and decreased patient survival.

6. Role of ROS as Signaling Molecules in Angiogenesis

ROS can act as a signaling molecule for activation of diverse signaling pathways by oxidation of reactive cysteine on the specific target molecules including kinases, phosphatases [28–30], redox sensitive transcripition factors [112], cell cycle regulators [113].

NADPH oxidase is activated by numerous stimuli including VEGF, EGF, cytokines, shear stress, hypoxia and G-protein coupled receptor agonists including Ang II in ECs [31]. VEGF binds to two tyrosine kinase receptors, VEGF receptor-1 (VEGFR1, Flt-1) and VEGFR2 in ECs. The mitogenic and chemotactic effects of VEGF in ECs are mediated mainly through VEGFR2 [4] which is activated through autophosphorylation of tyrosine residues in the cytoplasmic kinase domain. This event is followed by activation of downstream signaling pathways such as mitogen-activated protein kinases, Akt and eNOS, which are essential for EC migration and proliferation [4]. VEGF stimulation increases ROS production via activation of Rac1-dependent NADPH oxidase in ECs [11, 21, 23, 109, 114, 115]. We and others have shown that ROS are involved in VEGF-induced VEGFR2 autophosphorylation in ECs [11, 23, 114] (Figure 3). Ang-1 also stimulates ROS production through activation of Rac1-dependent, Nox2-based NADPH oxidase through Tie-2 receptor (Tie-2 R), which is required for EC chemotaxis [25]. ROS are also important for VEGF-induced cSrc activation, phosphorylation of VE-cadherin and Akt, thereby stimulating angiogenesis in ECs [21, 89, 115]. Evidence suggests that VEGFR2-mediated signaling is temporally and spatially controlled and that NADPH oxidases are localized within discrete subcellular compartments, which is required for localizing ROS production and activation of specific redox signaling events [32, 116].

The signaling properties of ROS are due, in part, to reversible oxidative inactivation of redox-sensitive target proteins including protein tyrosine phosphatases (PTPs) [28–30]. SHP-2, one of the SH2-containing PTP, can be inactivated by ROS in PDGF-treated Rat-1 cells, which is associated with autophophorylation of PDGFR leading to MAPK activation [117]. Several PTPs including SHP-1, SHP-2 and low molecular weight PTP (HCPTPA) inducibly associate with VEGFR2 after VEGF stimulation [118–120]. HCPTPA overexpression inhibits VEGF-induced VEGFR2 autophosphorylation but its endogenous role has not been demonstrated [120]. SHP-1 mediates TNF-induced inhibitory effect on VEGFR2 phosphorylation without affecting VEGF-induced responses in ECs [121]. SHP-2 negatively regulates VEGFR2 signaling in ECs which are cultured only on type I collagen [122]. High cell density-enhanced PTP1 (DEP-1)/CD148 attenuates phosphorylation of VEGFR2 in contact-inhibited confluent ECs [123]. A small molecule inhibitor of PTP1B enhances VEGF-induced VEGFR2 autophosphorylation, migration and proliferation of EC as well as neovascularization in a mouse matrigel model [124]. Ang-1 stimulates association of SHP-2 to the phosphorylated Tie-2 R in ECs [125], which in turn inhibits PI3 kinase-dependent signaling pathways involved in EC migration.

Tumor suppressor PTEN dephosphorylates phosphatidylinositol 3,4,5-triphosphate, a product of the PI3 kinase (PI3K) reaction, and is critically involved in a wide variety of human tumors [126]. ROS regulate EGF-induced increase in VEGF and HIF-1α expression through activation of PI3K/Akt/p706K pathway which is involved in tumorigenesis and angiogensis in ovarian cancer cells [127]. Although underlying mechanisms remain unclear, it is tempting to speculate that ROS-dependent oxidative inactivation of PTEN [128] may be involved in this response. Low molecular weight PTPs are oxidized and inactivated by ROS during cell adhesion to matrix in fibrobrast [129]. ROS derived from Rac1-induced NADPH oxidase inactivate PTP-PEST at focal contacts, thereby promoting membrane ruffling and endothelial migration [103]. Thus, it is likely that PTPs and PTEN are reversibly oxidized and inactivated by angiogenesis growth factor-induced ROS, thereby promoting RTK-mediated redox signaling linked to angiogenesis (Figure 3).

7. Redox-sensitive Transcription Factors and Genes involved in Angiogenesis

Transcription factors and genes involved in angiogenesis are regulated by ROS. These redox-sensitive transcription factors and genes include hypoxia inducible factor-1 (HIF-1), Ref-1,

p53, NF-κB and the ETS transcription factor, Ets-1 as well as VEGF, MMP, cyclooxygenase-2 (COX-2), urokinase plasminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1) (Figure 3).

HIF-1

In response to tumor hypoxia, many angiogenesis-related genes including VEGF and erythropoietin are upregulated by HIF-1, which is a heterodimeric transcription factor composed of HIF-1α and HIF-1β subunits [130–132]. HIF-1 activates the transcription of many genes involved in multiple aspects of tumor growth including angiogenesis, cell survival, and invasion. High levels of HIF-1 expression are observed in many human cancers, and are correlated with tumorigenesis [133]. ROS derived from NADPH oxidase are also involved in induction of HIF-1α under normoxia and hypoxia in vascular cells [134–136]. Görlach et al. [137] reported that overexpression of Rac1 increases HIF-1 expression through ROS. Thus, Rac1/Nox/ROS pathways play an important role for upregulation of HIF-1 and VEGF expression in response to VEGF and hypoxia. A cytochrome b-type NADPH oxidoreductase or mitochondria also produce ROS under hypoxia. As mentioned, ROS are involved in upregulation of HIF-1α and VEGF protein expression through activation of PI3K/Akt/p706K pathway or MEK/ERK pathway in cancer cells [138–142] Of interest, JunD, a member of the AP-1 family of transcription factors, promotes degradation of HIF-1α and reduces expression of VEGF by reducing Ras-mediated production of ROS, thereby inhibiting tumor angiogenesis [143]. These reports suggest that ROS play an important role for stabilizing HIF-1α protein expression, which may contribute to VEGF expression and tumor angiogenesis.

Ref-1

The redox state of cysteines is in part under the control of the redox factor 1 (Ref-1), a nuclear protein whose reducing activity also increases DNA binding activity of other transcription factors such as Fos, Jun, NF-κB [144], and p53 [145]

p53

p53 is an important intracellular mediator of the stress response including ROS and is now also recognized as a modifier of the angiogenic response [146]. p53 interacts with the HIF system but also has direct effects on angiogenesis regulators or interfere with translation mechanisms of angiogenesis factors and mediators such as VEGF [147] and FGF [148].

NF-κB

The redox-regulated transcription factor NF-κB is involved in NADPH oxidases-dependent tumor cell proliferation via regulating numerous genes involved in apoptosis, cell proliferation, metastasis and angiogenesis [149]. H_2O_2 induces NF-κB-dependent IL-8 expression in ECs, which contributes to the angiogenic phenotype [35]. NF-κB is also constitutively expressed in numerous malignancies [149], and is predominantly activated in adenoma and adenocarcinoma cells which express abundant Nox1, suggesting that Nox1 stimulates NF-κB-dependent pro-inflammatory pathways in colon tumors [150].

Ets

Ets-1 is a transcriptional mediator of vascular inflammation and remodeling [151]. H_2O_2 stimulates induction of the transcription factor Ets-1, which are involved in EC proliferation and tube formation [13] as well as expression of chemokine monocyte chemoattractant protein-1, the adhesion molecule vascular cell adhesion molecule-1, and the PAI-1 [151]. Most recently, Ni et al. [152] demonstrated that Ets-1 is a critical transcriptional regulator of angiotensin II-mediated ROS generation and the induction of NADPH oxidase subunit $p47^{phox}$. Thus, positive

feed-forward mechanism may exist whereby ROS induce Ets-1 expression and activated Ets-1 increases expression of ROS-generating enzyme.

MMP

MMP is another ROS-regulated molecule involved in the progression of tumor-induced angiogenesis [19]. The presence of MMPs is essential for endothelial cell ingression into the tumor tissue because they degrade the extracellular matrix of the tumor cells and free the way for migrating ECs [153]. ROS can activate MMP-9 [154] and VEGF stimulates MMP-1 and 2 expression in ECs [155]. Wartenberg and his colleagues demonstrated that confrontation cultured ECs differentiated from embryonic stem cells invade tumor tissue, which results in tumor vascularization and growth with an increase in ROS [156]. This data strongly suggests that tumor-induced angiogenesis requires the presence of ROS for EC invasion. In parallel with this phenomenon, up-regulation of MMPs was observed, which was abolished in the presence of free radical scavengers [157]. Thus, ROS-dependent MMP expression is a prerequisite for vascular growth within the tumor tissue with subsequent tumor expansion (Figure 3).

COX-2

COX-2 is a key enzyme in the synthesis of prostaglandins and thromboxans and is highly upregulated in tumor cells and angiogenic ECs during tumor progressioin, and plays an important role in tumor angiogenesis. Involvement of NADPH oxidase-derived ROS in COX2 induction has been demonstrated [158–163].

Urokinase plasminogen activator (uPA)

uPA, its receptor (uPAR) and PAI-1-mediated signaling are involved in tumour cell invasion, survival, angiogenesis and metastasis [164–167]. These genes are upregulated by ROS [168, 169]. Silensing uPA promoter with siRNA inhibits tumor cell invasion and angiogenesis *in vitro* as well as prostate tumor growth and lung metastasis in a bioluminescence tumor/metastasis model [170].

8. Prevention of angiogenesis by dietary antioxidants

Because ROS play an important role in tumor angiogenesis, treatment with dietary antioxidants such as food phytochemicals which have antioxidant capacity seems to be a promising anti-angiogenic strategy. Especially dietary polyphenols are involved in protection against not only cardiovascular risk factors such as atherosclerosis but also cancer angiogenesis by inhibiting oxidative stress. Recent studies have indicated that both red wine and green tea polyphenols prevent effectively the thrombin-induced activation of MMP-2 in vascular smooth muscle cells [171]. VEGF expression and its release are prevented by red wine polyphenols [172]. In addition, green tea polyphenols and epigallocatechin-3-gallate reduce VEGF expression in several types of cancer cells by inhibiting epidermal growth factor receptor (EGFR)-related signaling pathways [173, 174] Furthermore, natural polyphenols can inhibit migration and proliferation of vascular cells [171]. Consistent with these observations, either red wine polyphenols, green tea polyphenols or epigallocatechin-3-gallate inhibits angiogenesis in ECs [175] and in animal model of angiogenesis [172, 176–178]. Thus, dietary antioxidants appear to be effective for treatment of tumor angiogenesis. Understanding the molecular mechanisms of their action should provide basis to design more effective anti-angiogenic drugs targeted to various cancers.

9. Conclusion

Accumulating evidence suggest that ROS derived from NADPH oxidase play an important role in physiological and pathological angiogenesis. ROS function as signaling molecules to mediate various angiogenic-related responses such as cell proliferation, migration and

angiogenic gene expression in ECs and cancer cells. However, significant work remains to be performed; 1) to define the role of each Nox and its regulatory subunits in tumor angiogenesis; 2) to determine the activation mechanisms of NADPH oxidase by various angiogenesis factors; and 3) to identify molecular targets of oxidase-derived ROS in signaling pathways involved in angiogenic switch in various cancer cells. The development of specific inhibitors of NADPH oxidases and redox signaling components (kinase, phosphatase, transcription factors and genes) as well as understanding the mechanism by which dietary antioxidants inhibit angiogenesis could provide useful therapeutic strategies for treatment of various angiogenesis-dependent pathophysiologies such as cancer.

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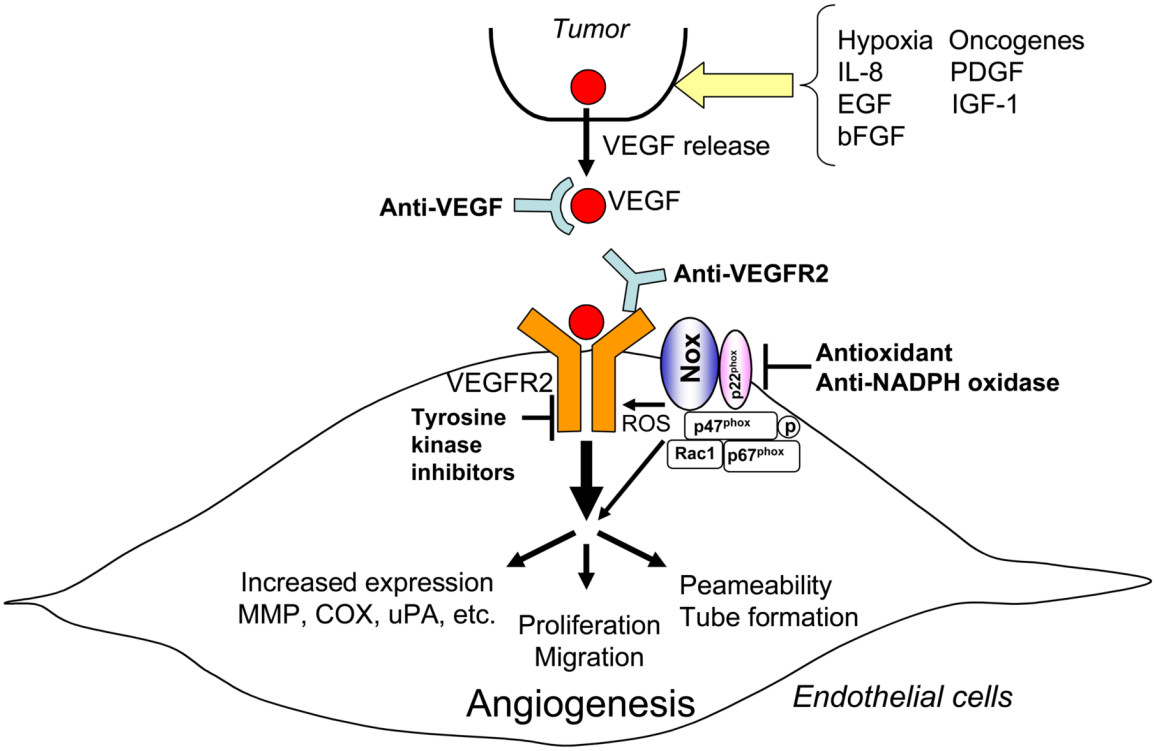
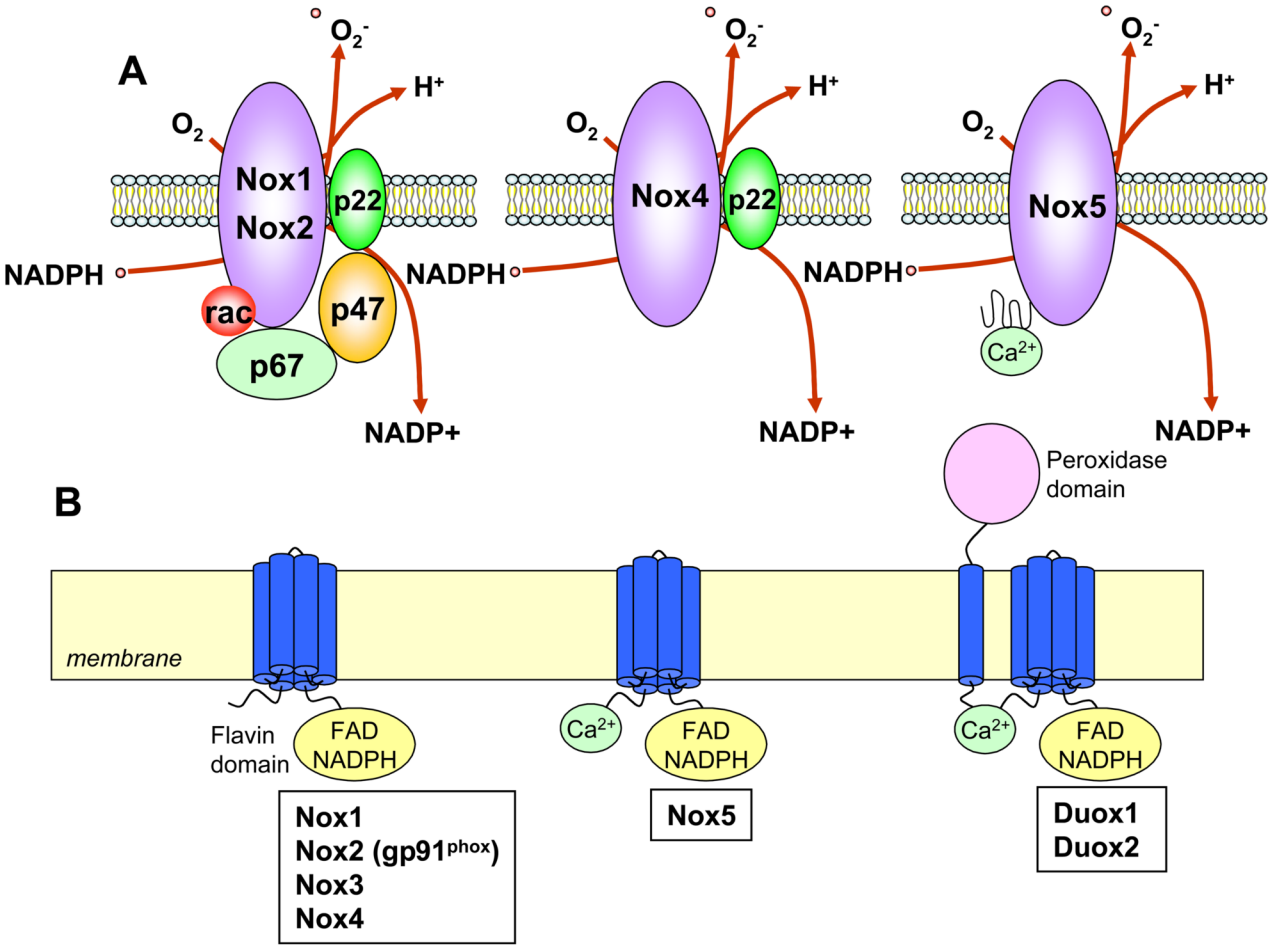


Figure 1. Anti-angiogenic therapy targeting NADPH oxidase (ROS)-dependent VEGF signaling

Progression to a growing tumor is characterized by induction of proangiogenic factors such as vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), and VEGF receptors (VEGFR) in the growing endothelial cells (ECs). VEGF is induced from tumor and stimulates permeability, proliferation, migration and tube formation of ECs primarily through the VEGFR type2 (VEGFR2). The conversion to the angiogenic phenotype in previously dormant tumors is known as the “angiogenic switch”. Thus, anti-angiogenic therapy is essential strategy for the treatment of tumor. In addition to VEGF and VEGFR2, ROS and NADPH oxidase are potential therapeutic targets for the treatment of tumor angiogenesis.



Shematic diagram of the structure of endothelial NADPH oxidase (A) and transmembrane topology of Nox and Duox enzymes (B)

Figure 2. A, Shematic diagram of the structure of NADPH oxidase in ECs

gp91^{phox} (Nox2) and its homologues (Nox1, Nox4 and Nox5) and cytosolic components p47^{phox}, p67^{phox} and small GTPase Rac1 have been identified in ECs. **B, Transmembrane topology of Nox and Duox enzymes.** The predicted transmembrane α-helices contain conserved histidine residues which comprise binding sites for haems. The carboxyl-terminal domain folds within the cytoplasm and binds to flavin adenine dinucleotide (FAD) and NADPH. The enzymes catalyze the transfer of electrons from NADPH to molecular oxygen, to form O_2^- across the membrane. The amino-terminal calcium-binding domain of Nox5 and Duox enzymes are also predicted to be on the cytosolic side of the membrane. Additional transmembrane α-helix of the Duox enzymes at the amino-terminus localize the peroxidase domain to the opposite side of the membrane, where it can use ROS generated by the catalytic core to generate more powerful oxidant species that then oxidize extracellular substrates.

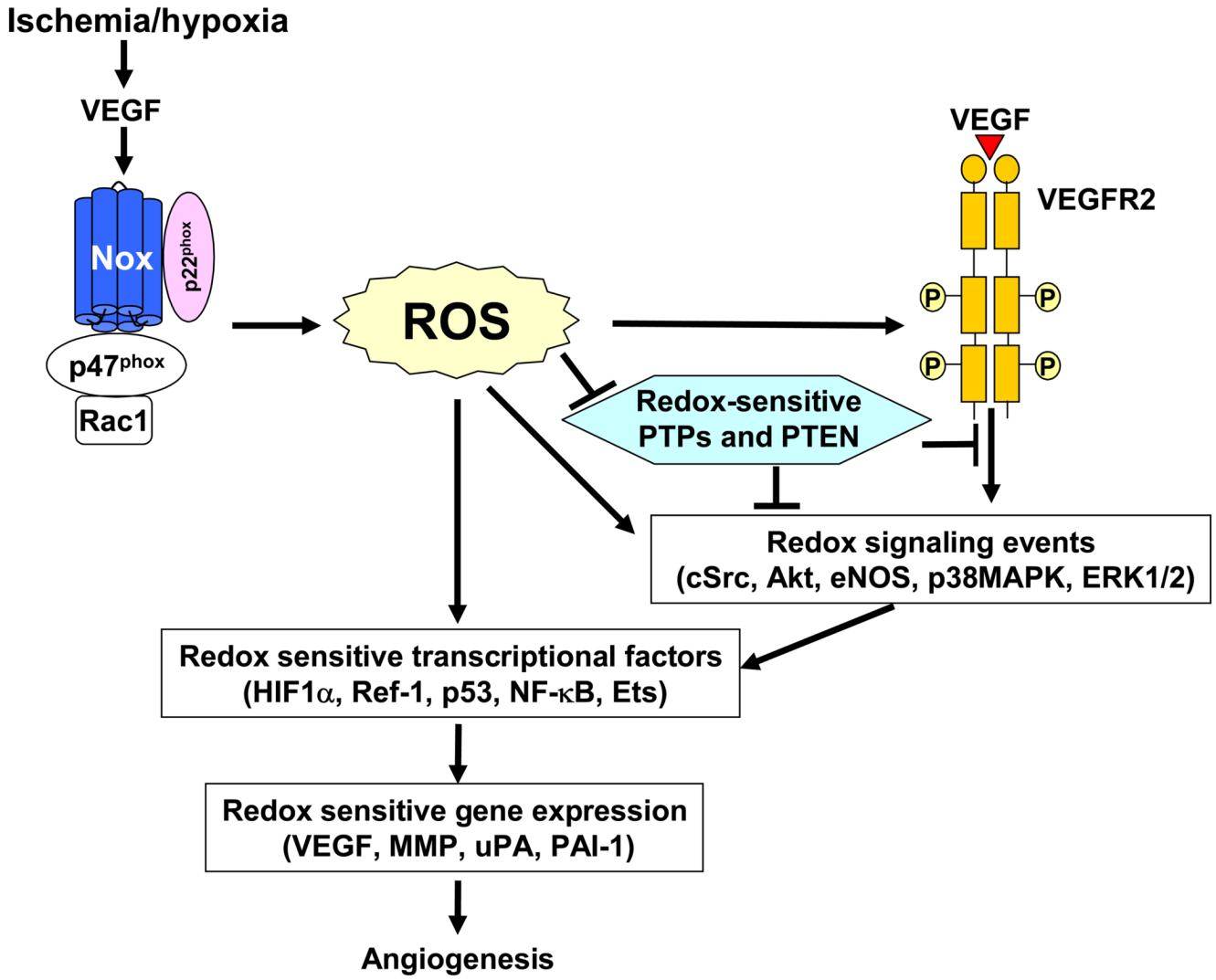


Figure 3. Role of ROS derived from NADPH oxidase in VEGF signaling linked to induction of transcription factors and genes involved in angiogenesis

Ischemia/hypoxia stimulates induction of VEGF which stimulates NADPH oxidase to produce ROS, thereby inducing oxidative inactivation of protein tyrosine phosphatases (PTPs) and PTEN to promote VEGFR2 autophosphorylation and downstream redox signaling events or directly activating redox signaling kinases. These events are converged and integrated to induce various redox sensitive transcriptional factors and gene expression, which are involved in angiogenesis.