Molecular Regulation of Tumor Angiogenesis and Perfusion via Redox Signaling

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1. Preface

Over the past decade, it has become clear that the gene mutations that initiate carcinogenesis are an inevitable aspect of aging. By age 70, the incidence of cancerous lesions in the thyroid has been estimated to approach 100%, and a high percentage of histologically positive cancers can also be detected in colon, prostate, and breast.[1] Yet, only a small percentage of these carcinomas in situ ever progress to cause frank disease, creating a dilemma for determining which will eventually become life-threatening malignancies that require treatment. Lifestyle changes may decrease the incidence of some of these cancers, and chemoprevention research will hopefully further decrease their initiation. However, for the

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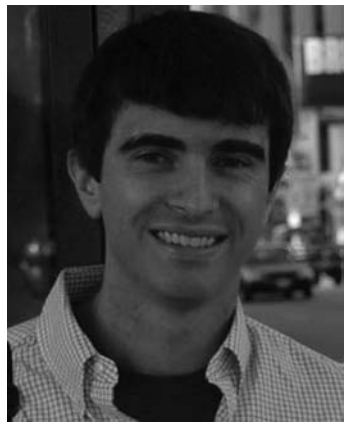

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near future, more benefit may come from understanding what distinguishes malignant cancer from a benign carcinoma in situ.

As an alternative to conventional cytotoxic chemotherapy, biological therapies that target physiological processes required for malignant tumor growth have attracted much recent attention. Pioneering studies by the late Dr. Judah Folkman introduced the concept that recruitment of a blood supply is critical to solid tumor growth.[2] Benign lesions show the enhanced cell proliferation characteristic of cancer but fail to grow beyond a size limit set by the ability of nutrients and oxygen to diffuse into the lesion. In this state, cancerous cells can exist for many years in a self-limiting cycle of proliferation and death.[3]

One way that such premalignant lesions progress toward malignant cancers is to initiate recruitment of blood vessels, a process known as the angiogenic switch. Much progress has been made toward understanding the molecular basis for this switch.[4] In addition to increasing production of proangiogenic factors, the angiogenic switch requires shutting off expression of endogenous antiangiogenic factors. A number of the identified pro- and antiangiogenic factors are proteins. Among the former, vascular endothelial growth factor (VEGF) and its receptor VEGFR2 have been successfully targeted by pharmaceutical companies using VEGF-neutralizing antibodies and small-molecule kinase inhibitors of the receptor. Several of these agents are now FDA-approved antiangiogenic drugs and show efficacy to extend the survival of cancer patients.[5−7] The hope was that these drugs would convert malignant cancer to a survivable benign disease. This ideal has been realized in some animal tumor models,[8,9] but in clinical practice, antiangiogenic drugs only extend the lifespan of advanced cancer patients on average by less than 1 year. This may be due in part to the plasticity of tumors to induce alternate proangiogenic factors that bypass the targets of the existing drugs. Therefore, we need either to develop drugs to target all possible angiogenic factors produced by the tumor or to identify conserved aspects of the signal transduction pathways used by these factors that can be the targets for universal angiogenesis inhibitors. Such targets are viewed by systems biologists as signaling nodes.[10]

This review focuses on a group of angiogenic signaling nodes that are of increasing interest as targets for antiangiogenic drug development. Subsequent to its discovery as a paracellular signaling molecule that is responsible for endothelium-dependent vasodilation, nitric oxide (NO) was found to also be a mediator of proangiogenic signaling by VEGF and other angiogenic growth factors.[11−13] We will discuss the sources and downstream targets of NO that play critical roles in angiogenesis and its regulation by the endogenous angiogenesis inhibitor thrombospondin-1 (TSP1). In addition to NO, two other bioactive gases are becoming recognized as important regulators of angiogenesis: carbon monoxide (CO) and hydrogen sulfide (H_2S). Two additional redox-active molecules, superoxide ($O_2^{\bullet-}$) and hydrogen

peroxide (H_2O_2), play important roles in angiogenic signaling, both directly and through their chemical reactions with NO. We will discuss the mechanisms be which redox signaling regulates angiogenesis and prospects for targeting these signaling pathways for therapeutic prevention and control of tumor angiogenesis and growth.

Finally, studies in animals have shown that angiogenesis inhibitors can synergize with other standard modes of cancer treatment. A number of clinical trials are ongoing using angiogenesis inhibitors in combination with chemotherapeutics and radiotherapy.[14] We will discuss how aspects of redox signaling may contribute to these synergistic activities and may guide the optimization of such therapeutic combinations.

2. Introduction to Angiogenesis

Angiogenesis is one of several processes that form new blood vessels in higher animals, but it has received the most research attention and popular interest because of its important roles in cancer and wound repair. During early embryogenesis, the first capillary networks form by a process known as vasculogenesis. Cells in the mesoderm differentiate into vascular endothelial cells and spontaneously connect to form a network of tubes known as a vascular plexus.[15] In contrast to angiogenesis, embryonic vasculogenesis occurs in the absence of blood flow. This primitive vascular network connects to primitive arteries and veins in the embryo, which establishes blood flow in the developing tissue. The directional flow is one signal that can promote differentiation of the vascular plexus into a hierarchical network of arteries, arterioles, capillaries, venules, and veins.[16] This differentiation process is known as arteriogenesis. Arteriogenesis is also directed by growth factors released from growing nerves in the embryo, which results in the parallel organization of blood vessels and nerves noted by early anatomical studies.[17]

During later development and in adult tissues, angiogenesis plays a major role in new blood vessel formation. Angiogenesis is defined as the formation of new blood vessels from an existing perfused vessel bed. This occurs by sprouting of endothelial cells in the vessel wall, either arterial or venous vessels depending on the soluble factors present,[18,19] which degrade and invade through the underlying basement membrane barrier and then further invade through the underlying extracellular matrix. As the leading cell moves forward, the following endothelial cells proliferate and differentiate to form a luminal space. The leading cell eventually finds another vessel, with which it fuses to establish a patent perfused vessel. Further cycles of this process accompanied by arteriogenesis produces a mature vascular network.

In addition to endothelial cells, mature blood vessels require supporting smooth muscle cells. During development, these can be recruited from mesenchymal stem cells or from bone-marrow-derived cells. Arterial vessels develop a thick layer of well-organized vascular smooth muscle cells (VSMCs) to accommodate the greater hydrostatic pressure in the arterial vasculature. These arterial smooth muscle cells, as will be discussed in greater detail below, also play an important role in adjusting blood flow to specific tissues in response to changing metabolic needs. Veins also have well-organized smooth muscle layers, but thinner than those in arteries. The VSMCs in capillaries are known as pericytes. In contrast to large vessels, capillary endothelial tubes are not completely covered by pericytes. Rather, the pericytes play important roles in capillary stability and function by secreting factors that regulate endothelial cell function and through direct contact with the adjacent capillary endothelium.[20]

Because of the positive hydrostatic pressure in perfused vessels, a net flow of water, ions, and small solutes constantly occurs across the vessel wall. This is opposed by an osmotic gradient resulting from the lower macromolecular solute concentration in the interstitial space, but nonetheless, net fluid movement occurs from perfused vessels into the underlying tissue. To maintain a constant blood volume, higher animals have a second vascular network, the lymphatics, that return this fluid to the cardiovascular system.[21] Lymphatics are a blind ended tree of specialized vascular cells, which form by a process known as lymphangiogenesis.

It has recently become clear that angiogenesis is not the only mechanism responsible for neovascularization of tumors and wounds in the adult.[22] In adult tissues, vasculogenesis is mediated by recruitment of circulating endothelial precursor cells that differentiate from hematopoietic stem cells in the bone marrow. These along with specialized monocytic stem cells cooperate to form new vessels at sites of injury and in some cancers. The relative contribution of angiogenesis versus vasculogenesis to tumor neovascularization is a subject of active current debate, but it is clear that some tumors depend significantly on bone marrow precursor recruitment, whereas this plays a minimal role in others.[23,24] Likewise, the role of lymphangiogenesis in tumor growth appears to be quite variable, with a subset of tumors being highly dependent on this process.[25]

This review focuses on the role of redox signaling in angiogenesis and angiogenesis inhibition, but the reader should remain aware that some proangiogenic factors can stimulate vasculogenesis, lymphangiogenesis, and arteriogenesis as well as angiogenesis. Correspondingly, angiogenesis inhibitors can often inhibit more than one of these processes. Therefore, the redox-signaling pathways discussed here have been initially defined and are best understood in the context of angiogenesis, but their true function may be more general.

3. Molecular Regulation of Angiogenesis

3.1. Vascular Endothelial Growth Factor Family

Angiogenesis is stimulated by several protein growth factors and steroids (Table 1). Among these, the vascular endothelial growth factor (VEGF) family plays a major role. VEGF-A is essential for vasculogenesis and angiogenesis during embryonic development and similarly serves as a major angiogenic factor in tumors.[26] A VEGF-A heterozygous null mutant in mice, retaining one functional copy of the gene, is also embryonic lethal. Therefore, a precisely regulated level of VEGF is critical to this process. Studies using conditional VEGF-A knockouts in mice have further refined the function of VEGF-A in adult mammals. Conditional deletion of VEGF-A in muscle revealed an important role in exercise-induced angiogenesis.[27] Deletion in kidney podocytes resulted in proteinuria and thrombotic microangiopathy of the kidney.[28] Deletion in endothelial cells showed that, in addition to its paracrine stimulation of angiogenesis, VEGF-A is a critical autocrine factor for maintaining endothelial function in the adult.[29]

On the basis of its key role in angiogenesis, VEGF-A has attracted the most attention to date as a molecular target for inhibiting tumor angiogenesis.[30] The therapeutic antibodies

Table 1. Major Angiogenic Factors and their Receptors

factor	receptors	function	references
VEGF-A	VEGFR2, VEGFR1, Neuropilin-1	embryonic and adult vasculogenesis, angiogenesis, vascular permeability	26
VEGF-B	VEGFR1, Neuropilin-1	revascularization after myocardial infarction	34
VEGF-C	VEGFR3, Neuropilin-2, (VEGFR2)	embryonic lymphangiogenesis	390
VEGF-D	VEGFR3, Neuropilin-2	lymphangiogenesis?	390
PlGF	VEGFR1	tumor- or ischemia-induced vascularization	35, 391
FGF1 (acidic FGF)	FGFR1, FGFR2, FGFR3, FGFR4	tumor angiogenesis?	42
FGF2 (basic FGF)	FGFR1, $\alpha v\beta 3$ integrin, heparan sulfate proteoglycans	inflammation- and tumor-induced angiogenesis	42, 43
Angiopoietin-1 (Angiopoietin-2)	Tie2	remodeling of embryonic and adult vasculature, arteriogenesis	392
Adrenomedullin	calcitonin-receptor-like receptor/receptor activity modifying protein	embryonic and tumor angiogenesis	54, 393, 53
Interleukin-8/CXCL8	CXCR1 and CXCR2	tumor- and ischemia-induced angiogenesis	49
platelet-derived growth factor-B	PDGFR	pericyte recruitment required for embryonic and tumor angiogenesis	394
steroids	estrogen and glucocorticoid receptors, ER46	angiogenesis	102

Avastin (bevacizumab) and Lucentis bind to this growth factor and inhibit its activity. VEGF-A stimulates proliferation and motility of endothelial cells by binding to VEGF receptor-2 (VEGFR2) and, to a lesser extent, VEGFR1. VEGF-A also regulates vascular permeability by regulating endothelial cell−cell junctions and transcytosis through VEGFR2.[31,32] VEGFR2 is a tyrosine kinase receptor, and several therapeutic angiogenesis inhibitors, including the FDA-approved drugs sorafenib and sunitinib, act at least in part by inhibiting this kinase activity.[33]

In contrast to VEGF-A, VEGF-B is not required for embryonic vascular development, but it plays an essential role in the adult heart for revascularization of ischemic tissue following a myocardial infarct.[34] VEGF-B signals via VEGFR1, which is also the signaling receptor for placental growth factor (PlGF). Like VEGF-B, PlGF is not required for embryonic angiogenesis, but its absence impedes ischemia-induced angiogenesis and neovascularization of tumors in adult animals.[35,36]

VEGF-C and VEGF-D play roles in lymphangiogenesis and bind to VEGFR3 expressed on lymphatic endothelium. Deletion of VEGFR3 in mice results in death at embryonic day 10.5, before the emergence of the lymphatic vessels.[37] VEGF-C is essential for the formation of lymph sacs from embryonic veins, and its absence results in embryonic death of null mice.[38] In adult tumor-bearing mice, VEGF-C is required for lymphatic metastasis.[39] In contrast, VEGF-D is not required for embryonic development, possibly because VEGF-C can substitute for its function. However, transgenic expression of VEGF-D can complement some defects in a VEGF-C null mouse.[40] Therefore, the functions of VEGF-C and VEGF-D may be somewhat redundant.

3.2. Fibroblast Growth Factor Family

Fibroblast growth factor-2 (FGF2, also known as basic FGF) is another major mitogen and motility factor for endothelial cells. Like VEGF-A, FGF2 is sufficient to stimulate a full angiogenic response in a fertilized chicken egg or in the cornea of mice and rats, but it is not necessary for embryonic vascular development.[41] FGF2 stimulates angiogenesis via its Tyr kinase receptor FGFR1. However, engaging this receptor is not sufficient for signaling, which also requires heparan sulfate proteoglycans and $\alpha v\beta 3$ integrin as coreceptors.[42,43] Angiogenesis of some tumors is dependent on FGF2, prompting interest in antagonists of this factor and its receptor as therapeutic angiogenesis inhibitors.[44] Several agents that inhibit FGF2 binding to heparan sulfate proteoglycans have progressed to human clinical trials.

3.3. Angiopoietins

Angiopoietins are another family of growth factors that play essential roles in embryonic vascular development. Mice lacking either the angiopoietin-1 (Ang1) or its receptor Tie2 die between embryonic day 9.5 and 12.5 due to lack of remodeling of the primary vascular capillary plexus.[44,45] Ang1-signaling via Tie2 mediates remodeling and stabilization of cell−cell and cell−matrix interactions and plays a role in the recruitment of pericytes to the nascent vessels. Ang2(−/−) mice show defects in developmental remodeling of lymphatic vessels.[46,47] In contrast, the absence of Ang2 has more subtle effects on vascular development. Ang2(−/−) mice show defects in developmental remodeling of lymphatic vessels.[46,47] Lack of Ang2 also causes defective regression of the fetal vasculature in the vitreous body of the eye and disorganization and hypoplasia of the intestinal and dermal lymphatic capillaries. In adult mice, Ang2 was up-regulated in response to femoral artery ligation, and subsequent vascular remodeling in the ischemic limb was impaired by a specific Ang2 inhibitor, L1−10.[48] The authors proposed that Ang2 promotes arteriogenesis in this wound-repair model. In vitro evidence indicates that Ang2 has both stimulatory and inhibitory effects on angiogenic responses.

Interleukin-8 is another angiogenic cytokine.[49] Recent evidence suggests that it also mediates the stimulation of endothelial cell proliferation and migration by Ang1.[50] Thus, IL-8 may mediate some proangiogenic activities of Ang1. The angiopoietin family has been further expanded by the discovery of a family of related factors. These include mouse Ang3, human Ang4, and seven angiopoietin-like proteins.[51] Different members of this family have pro- or antiangiogenic activities in vitro, but their pathophysiological functions in angiogenesis in vivo are still under investigation.

3.4. Adrenomedullin

Adrenomedullin is a 52 amino acid peptide that is produced by proteolytic cleavage of its precursor preproadrenomedullin.[52] Adrenomedullin signals by binding to a cell-surface seven-transmembrane G-protein-coupled receptor. Adrenomedullin is highly expressed in certain cancers and is an important angiogenic factor for these tumors.[53] Deletion

of adrenomedullin in mice is embryonic lethal at day 13.5−14 due in part to disorganization of endothelial cells and their underlying basement membrane.[54] Therefore, adrenomedullin is necessary for embryonic vascular development.

3.5. Steroids

Steroid hormones also play important roles in angiogenesis.[55] Glucocorticoids are an essential growth factor for endothelial cells in vitro. Estrogens also stimulate endothelial cell growth in vivo and induce NO signaling.[56] Effects of estradiol on endometrial angiogenesis in vivo include induction of VEGF, FGF2, Ang1, PlGF, eNOS, and soluble guanylate cyclase (sGC).[57] Therefore, estrogens coordinately induce upstream and downstream elements of NO/cGMP signaling under conditions where they stimulate angiogenesis. The estrogen metabolite 2-methoxyestradiol has been tested in clinical trials as an angiogenesis inhibitor.[58] However, its antitumor activity may be partially independent of inhibiting angiogenesis or antagonism of estrogens.[57]

3.6. Proangiogenic Factors in Cancer

The diversity of angiogenic factors combined with the ability of cancers to change their expression of specific factors creates a challenge for therapeutic control of tumor angiogenesis. It is clear from experimental animal tumor studies and from clinical experience with existing angiogenesis inhibitors in cancer patients that tumors become resistant to specific angiogenesis inhibitors.[59] Thus, the current FDA-approved drugs typically extend cancer patient survival by 3−6 months, but long-term control of cancer growth by angiogenesis inhibitors as single agents is rare. Current clinical trials are exploring whether combinations of angiogenesis inhibitors are more effective, but at present such efforts are limited by the small number of inhibitors available. We do not know how many angiogenic factors a specific tumor can make, nor can we predict which will become dominant when one proangiogenic pathway is inhibited. In developing new antiangiogenic therapies, it is important to consider whether drug combinations can be found that will effectively inhibit all major tumor angiogenic factors or whether multiple angiogenic factors share some common downstream signaling pathway that would be a more effective drug target than the individual growth factors or their receptors.

4. Endogenous Angiogenesis Inhibitors

One way to approach this problem for cancer therapy is to study how angiogenesis is normally controlled during development and in adults and how this process becomes dysregulated in nonmalignant disease. Angiogenesis is highly controlled during embryonic development, with vascular density closely matched to the metabolic needs of a given tissue. In healthy adult individuals, angiogenesis is stimulated in a controlled manner during wound healing, cyclically in the uterine decidual lining during the menstrual cycle, and in specific muscle beds in response to exercise training. Yet, with increasing age and chronic conditions such as diabetes, the ability to stimulate angiogenesis becomes impaired, and tissues can become ischemic due to lack of adequate blood flow.[60] Conversely, excessive angiogenic responses are factors in growth of keloids and uterine fibroids.[61−63] It is becoming clear that these diseases cannot be explained merely by an excess or deficit in specific angiogenic factors but must be understood in terms of a net balance between angiogenic factors and endogenous angiogenesis inhibitors.

4.1. Thrombospondins

The first identified endogenous angiogenesis inhibitor was TSP1. A 140 kDa protein secreted by an immortalized hamster cell line was found to block angiogenesis in vivo, and its expression was controlled by a tumor-suppressor gene.[64] Loss of the tumor suppressor was accompanied by loss of the secreted protein and acquisition of angiogenic activity. The 140 kDa protein was purified and identified to be a proteolytic fragment of TSP1. Independently, two other groups reported in the same year that native TSP1 purified from platelets potently inhibits endothelial cell proliferation and chemotaxis stimulated by FGF2.[65,66] Subsequent studies confirmed the ability of TSP1 to inhibit angiogenesis in the rat cornea and the chick rat chorioallantoic membrane developmental angiogenesis assays.[64,67] This activity of TSP1 was extended to tumor angiogenesis by re-expressing TSP1 in a tumorigenic human melanoma cell line MDA-MDB-435.[68] Transfected clones overexpressing TSP1 formed slower-growing tumors in athymic mice that exhibited decreased densities of tumor blood vessels. Similar results in vivo were found when TSP1 was re-expressed in hemangioma, v-src-transformed NIH 3T3, cutaneous squamous cell carcinoma, glioblastoma, and hematopoietic tumor cell lines.[69] Furthermore, overexpression of TSP1 in the skin or mammary glands of tumor-prone mice suppressed formation and angiogenesis of carcinogen-induced premalignant epithelial hyperplasias and spontaneous mammary tumors, respectively.[70] Conversely, mice lacking TSP1 showed increased tumor growth when crossed with mice carrying cancer-promoting mutations in APC(Min/+) or mice lacking the tumor suppressor p53.[71] In a xenograft model of tumor dormancy, high expression of TSP1 was characteristic of nonangiogenic tumor cells that maintained prolonged dormancy when implanted in mice.[72]

A second member of the thrombospondin family, TSP2, was subsequently shown to be an angiogenesis inhibitor.[73] As found for TSP1, overexpression of TSP2 in tumor cells suppressed tumor growth and angiogenesis in mice,[74] and mice lacking TSP2 showed increased susceptibility to skin carcinogenesis and earlier switching to an angiogenic phenotype.[75]

4.2. Angiostatin

Many additional angiogenesis inhibitors have been and continue to be discovered (Table 2). Unlike TSP1, which is active in its native form, several of these inhibitors are derived by proteolysis of proteins that serve other physiological functions. Angiostatin is a proteolytic fragment of plasminogen, a precursor of an important protease for resolution of blood clots.[76] Plasminogen lacks antiangiogenic activity, but several of its Kringle repeats are potent inhibitors when released by proteolysis or expressed as recombinant proteins.[77]

4.3. Inhibitors Derived By Proteolysis of Extracellular Matrix

Several extracellular matrix collagens contain noncollagenous domains that have cryptic antiangiogenic activities.

Table 2. Endogenous Angiogenesis Inhibitors and Their Receptors

inhibitor	source	receptors	references
Thrombospondin-1		CD36, CD47, HSPG	150, 395, 153, 146
Thrombospondin-2		CD36	396
platelet factor-4		CXCR3-B, HSPG	397
Interferon-α, β		IFN-α/β receptor (IFNAR1/IFNAR2)	398, 399
chondromodulin-I, tenomodulin		?	400
pigment epithelium-derived factor		?	401
TIMP2		α3β1 integrin	402
Angiostatin	plasminogen	Fo-F1 ATPase, angiomotin, αvβ3	76, 403
endostatin	collagen XVIII	Nucleolin, other	78, 404
soluble VEGFR1 (sFlt1)	VEGFR1	decoy receptor for VEGF	26
Arresten, Canstatin, and Tumstatin	Type IV collagen	Integrins α1β1, α3β1, α6β1, αvβ3	81
Vasoinhibins	Prolactin, growth hormone, placental lactogen	?	405
Vasostatin	calreticulin	?	406
NK4	hepatocyte growth factor	c-Met, other?	407
Endorepellin	Perlecan	α2β1 integrin	82

The first identified inhibitor in this family was endostatin, which is a fragment of type XVIII collagen.[78] The protein was first identified as a circulating angiogenesis inhibitor in tumor-bearing mice. It has been expressed as a recombinant protein and progressed to human clinical trials as a therapeutic angiogenesis inhibitor.[79] Endostatin levels are controlled by the activities of proteases that release it from its parent collagen as well as by H_2O_2 and NO/cGMP signaling.[80] Similar inhibitors have been identified as proteolytic fragments derived from three subunits of basement membrane type IV collagen[81] and as the endorepellin fragment derived from the large basement membrane proteoglycan perlecan.[82]

5. NO Signaling in the Cross-Talk Between Proangiogenic and Antiangiogenic Factors

5.1. Proangiogenic Signal Transduction

Angiogenic growth factors typically engage plasma membrane receptors that have tyrosine kinase activity.[26,42,83] The signal transduction pathways activated by growth factors binding to these receptors can be quite complex (Figure 1). In addition to direct signaling through receptor kinase activation, lateral cross-talk involving other membrane components plays an important role. Syndecans and other heparan sulfate proteoglycans play important roles in FGFR dimerization, activation, and signaling. Cross-talk with neuropilins, integrins, and VE-cadherin is also important for VEGF receptor signaling.[84,85]

5.2. NO in VEGF Signaling

Autophosphorylation of VEGFR2 at Tyr^{951} mediates recruitment of T-cell-specific adapter (SH2 domain-containing protein 2A), which mediates recruitment of Src kinase.[26] Autophosphorylation of VEGFR2 at Tyr^{1175} recruits phospholipase Cγ and Shb. Phosphorylation of Tyr^{801} is required for recruitment of the p85 subunit of PI-3-kinase and consequent activation of Akt.[86] These proximal targets in turn activate a number of downstream targets, resulting in increased endothelial proliferation, motility, and permeability. Relevant to the present discussion, all three pathways have been implicated in VEGF-mediated activation of eNOS (Figure 1). Akt phosphorylates eNOS at Ser^{1177} and induces NO synthesis.[12,13] PLCγ signaling increases intracellular Ca^{2+}, which binds to calmodulin to further activate eNOS.[87] PLCγ signaling also activates AMP kinase, which further activates eNOS by phosphorylation at Ser^{1177}.[88] Src acts on at least two targets to activate eNOS. It phosphorylates heat shock protein-90 (Hsp90) at Tyr^{300}, which induces Hsp90 binding to eNOS and activation of NO synthesis.[89] Simultaneously, VEGF binding induces Src-dependent phosphorylation of eNOS at Tyr^{83}.[90] This phosphorylation is also required for eNOS activation.

In mice lacking eNOS, VEGF produces a decreased angiogenesis response relative to wild-type mice as assessed

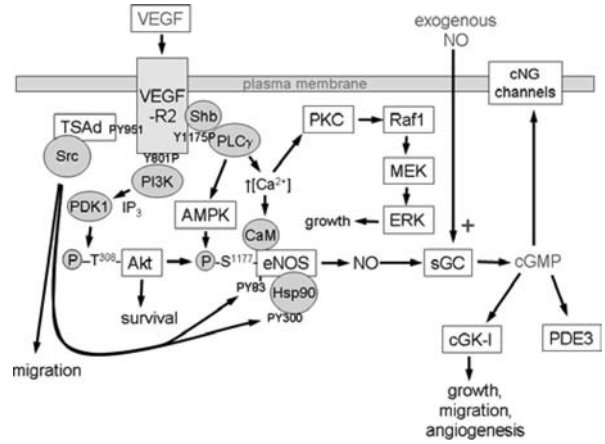

Figure 1. VEGF binding to VEGFR2 on endothelial cells activates its Tyr kinase activity and results in autophosphorylation of the receptor at several cytoplasmic sites. The phosphorylated Tyr serve as docking sites for specific signaling molecules. Phosphatidyinositol 3-kinase (PI3K) is recruited at phosphorylated Tyr^{801}, increasing inositol 1,4,5-triphosphate (IP3) formation, which in turn activates 3-phosphoinositide-dependent protein kinase-1 (PDK1) to phosphorylate and activate Akt, which phosphorylates human eNOS at Ser^{1177}.[12,13] This phosphorylation activates eNOS and decreases its calcium dependence. VEGFR2 phosphorylation at Tyr^{951} recruits TSAd and Src, which phosphorylates heat shock protein 90 (Hsp90) at Tyr^{300}, which induces Hsp90 binding to eNOS and activation of NO synthesis,[89] and phosphorylates eNOS at Tyr^{83}, which is also required for eNOS activation.[90] Phosphorylation of VEGFR2 at Tyr^{1175} recruits phospholipase-Cγ (PLCγ), which mobilizes intracellular Ca^{2+} and thereby further activates eNOS in a calmodulin (CaM)-dependent manner. PLCγ also increases AMP kinase (AMPK)-mediated eNOS phosphorylation at Ser^{117788}. NO produced by eNOS binds to the heme on soluble guanylate cyclase (sGC) to stimulate cGMP synthesis. cGMP in turn activates cGMP-dependent protein kinase (cGK-I) and cGMP-gated channels to regulate downstream targets that increase endothelial cell proliferation, migration, survival, and permeability.[127] cGMP also binds to and regulates several phosphodiesterases that terminate that cGMP signal or mediate cross-talk with cAMP signaling by hydrolyzing that second messenger. Additional parallel signaling through Src, Akt, and the protein kinase C-mitogen-activated protein kinase pathway (PKC-Raf1-MEK-ERK) synergizes with NO/cGMP signaling to support each of these endothelial cell responses.

using a type I collagen gel implanted under a cranial window.[91] iNOS null mice show less impairment in VEGF-induced angiogenesis, indicating that eNOS is the major mediator of the proangiogenic activity of VEGF in this assay. NO levels measured in the implanted gels were increased in the respective mice in proportion to the observed angiogenic responses. Therefore, eNOS mediates an NO-dependent angiogenic response to VEGF in vivo.

There are some discrepancies in the literature regarding NO-mediating VEGF-driven angiogenesis.[92] Some of these studies employ NO-donating molecules at supraphysiological concentrations as well as molecules that have other relevant reactivities in addition to directly releasing NO moieties. The reader is referred to work detailing the characteristics and use of NO donors for additional information on this.[93] NO is known to have biphasic dependence on concentration for a number of different aspects of cell proliferation and migration.[92,94,95] However, the responses triggered by NO donors at concentrations appropriate to activate sGC are consistently proangiogenic.

5.3. Broader Role of eNOS in Angiogenic Factor Signaling

VEGF is not the only angiogenic growth factor that signals via activation of eNOS.[96] Angiogenic responses to angiopoietin-1 are deficient in eNOS-null mice.[97] Angiopoietin-related growth factor (AGF) enhances blood flow in a mouse hindlimb ischemia model through induction of angiogenesis and arteriogenesis. In vitro, AGF increases NO production by human umbilical venous endothelial cells.[98] Furthermore, AGF did not restore blood flow to ischemic hindlimbs of either mice receiving the NOS inhibitor L-NAME or eNOS knockout mice. Therefore, NO may generally mediate proangiogenic activities of angiopoietin family members.

Although adrenomedullin null mice are not viable, heterozygous nulls survive to adulthood. Measurement of NO synthesis by perfused kidneys from adrenomedullin +/− mice showed an approximately 50% decrease in NO levels.[99] These mice displayed a higher resting mean arterial blood pressure that wild-type controls, indicating a functional deficiency in NO activity. This was verified to depend on adrenomedullin regulation of eNOS by treatment with a NOS inhibitor, which resulted in a smaller increase in blood pressure in heterozygous null mice than in wild type. Therefore, endogenous adrenomedullin is an important physiological inducer of vascular NO synthesis.

Lysophospholipids play broad roles in regulating cell behavior, and one of their targets is NO signaling. Sphingosine-1-phosphate (S1P) is an important prosurvival and chemotactic factor for endothelial cells. S1P activates eNOS in endothelial cells via the phosphatidylinositol-3-kinase (PI3K)/Akt pathway.[96] S1P-stimulated eNOS phosphorylation and NO production is blocked by inhibition of PI3K or Akt.[100] Similarly, S1P-stimulated capillary growth into subcutaneously implanted Matrigel plugs in mice was significantly reduced in mice that received the NOS inhibitor L-NAME. Lysophosphatidic acid also signals in endothelial cells through G protein-coupled Edg family receptors, and this signaling activates eNOS.[96]

FGF2 increases the expression of eNOS mRNA and the production of NO in human umbilical vein and calf pulmonary artery endothelial cells when cultured on three-dimensional fibrin gels.[101] However, other studies suggest that FGF2 stimulates angiogenesis by inducing expression of VEGF-A and its receptors,[42] so FGF2 may only indirectly regulate endothelial cell NO synthesis.

Proangiogenic signaling by estrogens involves both the conventional nuclear estrogen receptor-α and an N-terminal truncated isoform known as ER46, which is expressed in endothelial cells.[102] ER46 interacts with Src and mediates rapid activation of eNOS at the plasma membrane. In the presence of 17β-estradiol, phosphorylation of Src at Tyr^{419} is stimulated, and eNOS becomes phosphorylated at Ser^{1177}. ER46 colocalizes with caveolin-1 in endothelial cells, implying that ER46 associates with the eNOS-caveolin complex in endothelial cells.

Insulin and insulin-like growth factors have proangiogenic activities in vivo either alone or in conjunction with other growth factors.[103,104] Insulin signaling through the insulin receptor tyrosine kinase signals via PI3-kinase and activates eNOS by promoting Ser^{1177} phosphorylation as well as eNOS denitrosylation.[96]

5.4. Is NO Necessary for Angiogenesis?

Although the above studies establish that eNOS is necessary for stimulation of angiogenic responses by several growth factors, it remains unclear that NO itself is essential for angiogenesis. From the perspective of identifying therapeutic approaches to control pathological angiogenesis, this question needs to be addressed separately for developmental and pathological angiogenesis. The eNOS null mouse is viable and lacks major defects in its vascular anatomy except for a decrease in capillary density in the left ventricular myocardium, which is associated with abnormal aortic valve development.[105] Apart from this tissue, developmental angiogenesis does not appear to require eNOS. Note that this result does not prove that NO is not required for developmental angiogenesis since NO is produced by two other NOS isoforms. Mice lacking all three NOS isoforms also remain viable, and their hemodynamic parameters are similar to those of an eNOS null mouse.[106] The triple null mice lack any detectable NO synthesis, indicating that embryonic angiogenesis can occur in the complete absence of endogenous NO synthesis. Even in these triple nulls, however, some NO may be present due to nitrite/nitrate reductase activities that can derive NO from dietary nitrate/nitrite.[107] Furthermore, as discussed below, some additional gasotransmitters may activate sGC to compensate for the loss of NO signaling following complete NOS gene disruption, and VEGF is known to activate synthesis of some of these.

In adult mice, the absence of *NOS3* results in specific defects in angiogenic responses to several angiogenic factors including VEGF, Ang1, AGF, angiotensin-II, PlGF, and RANKL. A differential requirement for eNOS was reported for splitting versus sprouting angiogenesis.[108] These studies may begin to provide insight into why eNOS would be selectively required for certain types of pathological angiogenesis, but more research is needed to identify signaling pathways that are unique to pathological versus developmental angiogenesis.

A role for eNOS in tumor angiogenesis is supported by several studies.[109] Consistent with this data, treatment with L-NAME but not D-NAME inhibited neovascularization induced by the C3L5 murine mammary adenocarcinoma cell line.[110] Furthermore, intratumoral vessel density and morphology were specifically altered in B16 melanomas grown in eNOS null mice compared to either wild-type or iNOS null mice.[111] In light of the latter studies in mice, it is

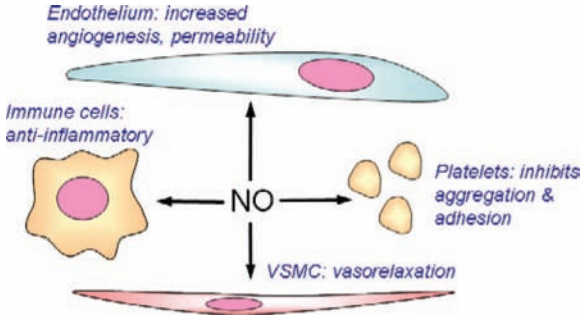

Figure 2. Nitric oxide acts on several target cells in blood vessels that are important for tumor biology. NO acts in endothelial cells to stimulate angiogenesis and increase vascular permeability. The former is important to neovascularization to support tumor growth. The latter contributes to the characteristic leakiness of the tumor vasculature. NO stimulates proliferation and migration of VSMC, which can contribute to angiogenesis. NO also relaxes VSMC, which can either increase or decrease tumor perfusion. NO inhibits platelet adhesion and aggregation. Platelet adhesion to circulating tumor cells plays an important role in metastatic spread of cancers.[408] Finally, NO has inhibitory activities for immune cells, which can limit host antitumor immunity.

interesting that polymorphisms in *NOS3* have been associated with increased risk for several cancers in humans.[112−117] A-786T>C polymorphism is particularly notable because it was associated with increased eNOS expression and significantly increased progression but not the incidence of prostate cancer.[118]

5.5. Nitric Oxide Signaling in Cardiovascular Physiology

To understand the implications of widespread eNOS regulation by proangiogenic and antiangiogenic factors, it is important to recognize the broader role of NO in vascular biology (Figure 2). A molecular understanding of the role of NO in mammalian vascular physiology began with the demonstration that organonitrates could activate sGC[119] and that NO caused vascular dilation.[120] The physiological relevance of these observations became obvious when NO was found to be produced by mammalian cells,[121] and endothelial cells lining of blood vessels were shown to be a source of endogenous NO production.[122] Near the same time, a specific calcium-dependent enzyme that synthesizes NO was isolated.[123] Endogenous NO functions to activate many critical survival-based pathways in multiple mammalian cell types. These processes derive from the ability of NO to activate the heme protein sGC. Under basal conditions (no NO), sGC catalyzes the production of the second messenger molecule cyclic guanosine monophosphate (cGMP) from GTP. This process is regulated by an allosteric 5-coordinate ferrous heme prosthetic group that is ligated to sGC by an axial Fe^{2+}−histidine bond.[124] NO binds to the Fe^{2+} of sGC, activating it over 200-fold. This is mechanistically dependent on NO induced loss of the axial Fe^{2+}−histidine bond.[124] This mode of binding is significant in light of the presence of other diatomic heme ligands (O_2 and CO) that could compete with NO but prefer to bind reduced hemes in a 6-coordinate fashion. Therefore, these molecules bind to sGC with a much lower affinity than NO (8 and 4 orders of magnitude, respectively)[125] and either do not activate sGC (O_2) or do so to a much lesser extent than NO (CO).[126] As an intracellular second messenger, cGMP produced by activated sGC amplifies the NO signal and functions to activate a number of signaling pathways that enhance vascular cell survival.[127] Physiologic levels of NO promote vascular cell proliferation and migration and vascular smooth muscle relaxation via the cGMP signaling pathway. In wound-healing environments, sustained NO signaling stimulates angiogenesis. At the level of organ systems, NO plays an important acute role to modulate arterial blood flow. By relaxing the contraction of the VSMC of arteries, NO increases vessel diameter, lowers arterial resistance, and enhances blood flow to tissues. Thus, endothelial cells lining blood vessels self-regulate their local arterial tone by continuously producing NO.

On the basis of its high diffusion coefficient, the signaling activity of NO is not restricted to its site of synthesis, and cell membranes are not a significant barrier. Apart from efficient scavenging by abundant heme proteins,[128] NO is free to activate downstream signaling throughout the local environment. Thus, until recently, it was unclear whether cells could regulate NO-driven responses other than by hydrolysis of the second messenger cGMP. A family of phosphodiesterases (PDEs) that hydrolyze cGMP clearly serve this function and have been major targets for pharmacological intervention to enhance NO signaling responses.[129] Yet it remains a paradox that exogenous NO via inhaled gas or nitroglycerin as a stable form of deliverable NO remains physiologically active, but inhibition of PDE activity does not dramatically alter blood pressure.[130,131] These empiric findings suggest that NO signaling is subject to additional regulatory controls.

5.6. Angiogenic Factors as NO-dependent Vasodilators

Despite the broad recognition that NO is a pleiotropic regulator of cardiovascular physiology, the idea that angiogenic factors and their inhibitors could have acute cardiovascular effects has attracted little attention. This is slowly changing as the frequency of hypertensive and prothrombotic side effects of therapeutic angiogenesis inhibitors has become clear.[132−134,28] The ability of VEGF to induce NO-dependent relaxation of arterial segments was reported in 1993,[135] but direct demonstration of an acute hypotensive activity of VEGF was only confirmed recently.[136] This acute vasodilator activity is not unique to VEGF signaling through VEGFR2 because similar vasodilator activity was found for PlGF.[137] However, PlGF exhibited its NOS-dependent vasodilator activity via VEGFR1 rather than VEGFR2. Furthermore, adrenomedullin acts as a peripheral and coronary vasodilator by stimulating NO signaling.[138]

5.7. Thrombospondin-1 blocks NO-driven Angiogenesis

In studying the antiangiogenic activity of TSP1 we discovered that the NO/cGMP pathway is an important target of its signaling in endothelial cells.[139] An in vitro assay model was employed that simulates wound healing. Fresh skeletal muscle biopsies are implanted into a 3D matrix of type I collagen and incubated with growth medium. Within 72 h under these conditions, a predictable degree of vascular cell invasion and migration through the matrix occurs. Tissue samples from TSP1 null mice under the same growth conditions demonstrate an enhanced angiogenic response compared to those from wild-type mice. More importantly, NO-stimulated angiogenic response is always dramatically greater in explants from null animals compared to wild type. Thus, endogenous levels of TSP1 are sufficient to limit NO-

driven angiogenesis. NO-stimulated increases in proliferation, matrix adhesion, and migration of primary human vascular endothelial cells are all potently blocked by TSP1. Concentrations of TSP1 as small as 10 pM are sufficient to block prosurvival responses to NO in endothelial cells. These amounts are well within the demonstrated concentrations of TSP1 in plasma. Similarly, primary endothelial cells from wild-type and TSP1 null mice demonstrate that endogenous TSP1 limits NO-stimulated increases in cell proliferation and migration.

Increased numbers of VSMCs were found in the vascular cell outgrowth from the muscle explants of TSP1 null mice, suggesting that TSP1 also limits NO-driven responses in VSMCs. The activity of TSP1 to inhibit NO-driven responses was confirmed in human aortic VSMC, and murine primary aortic VSMC from TSP1 or CD47 null mice were found to have elevated resting and NO-stimulated cGMP levels.[140] Whereas TSP1 typically has opposing effects on endothelial and VSMC proliferation in the absence of NO,[65,141−143] coordinate regulation of VSMC and endothelial cells in the presence of NO may facilitate a balance of both cell types required for angiogenesis.

5.8. NO Signaling is Regulated Through the TSP1 Receptors CD36 and CD47

TSP1 was first identified in 1971 as a major secretory component of activated platelets,[144,145] and its antiangiogenic activity was recognized in 1990.[64−66] Different domains of TSP1 are now known to have pro- or antiangiogenic activities by engaging at least nine different receptors on endothelial cells.[146−150] These include several integrins, heparan sulfate proteoglycans, LDL receptor-related protein, CD36, and CD47.

In chemotaxis assays, CD36 deficient endothelial cells were insensitive to inhibition by TSP1, and re-expression of CD36 restored the inhibitory effect of TSP1.[150] Lack of TSP1 activity to inhibit corneal angiogenesis in CD36-null mice further indicated that the antiangiogenic activity of TSP1 requires CD36 binding.[151] A recombinant portion of the protein that engages CD36 (its type 1 repeats, Figure 3), a CD36-binding peptide derived from this domain (VTCGGGVQKRSRL), and certain CD36 antibodies (clone SMΦ) can mimic TSP1 by inhibiting NO-stimulated responses in vascular cells in vitro[139,140] and angiogenesis in vivo.[150,152] Therefore, engaging CD36 is sufficient to inhibit NO signaling. However, TSP1 remains a potent inhibitor of NO signaling in CD36 null vascular cells and muscle explants.[153] Thus, CD36 interaction with TSP1 is not necessary to block NO signaling in vascular cells.

In contrast, TSP1 failed to inhibit NO-driven angiogenesis in tissue explants from CD47 null mice, and NO-driven responses in vascular cells from CD47 null mice were insensitive to inhibition by TSP1.[153] Thus TSP1 blockade of physiologic NO signaling in vascular cells requires CD47. Under basal conditions, vascular cells from CD47 null mice always have elevated cGMP levels compared to wild-type cells. Similarly, temporarily suppressing CD47 expression increases basal cGMP levels.[154] Thus, TSP1 signaling via CD47 constantly limits the sensitivity of sGC to activation by NO and thereby sets basal intracellular levels of cGMP. TSP1 engages CD47 via the C-terminal domain of the protein (Figure 3). A recombinant protein containing this domain mimics the potent inhibitory effects of the whole protein on NO-stimulated vascular cell responses. Treating vascular cells

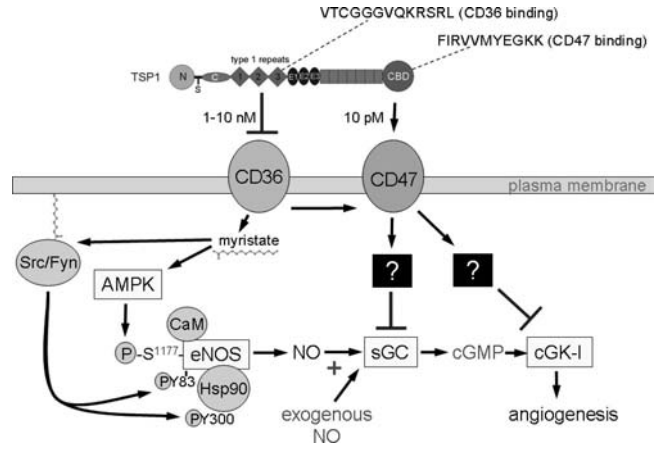

Figure 3. Thrombospondin-1 (TSP1) binds to two receptors on endothelial cells that inhibit NO signaling. The second and third central type 1 repeats of TSP1 contain sequences with known CD36-binding activities. The intact protein, recombinant type 1 repeats, or synthetic peptides derived from the active repeats bind to CD36 and inhibit uptake of myristate via this plasma membrane fatty acid translocase. Myristate mediates membrane anchoring of a number of signaling proteins including the Src kinases. Membrane anchoring of Src and other undefined targets leads to increased activation of eNOS, which is inhibited by TSP1.[160] Simultaneously, myristate activates eNOS in an AMP kinase (AMPK)-dependent manner,[158] which may also be inhibited by TSP1. Inhibition of NO signaling via CD36 requires concentrations of TSP1 that exceed those normally circulating in plasma but can be found in plasma of some cancer patients.[176,177,409] The more potent TSP1 inhibitory pathway involves the signaling receptor CD47. Two CD47-binding sequences have been identified in the C-terminal domain of TSP1.[155] Engaging CD47 signals through undefined pathways that simultaneously inhibit activation of soluble guanylate cyclase (sGC) and cGMP-dependent protein kinase (cGK-I).[157,410] These pathways inhibit NO signaling due to either endogenous or exogenous NO.

with a CD47-binding peptide from the C-terminal module of TSP1 (p7N3, FIRVVMYEGKK)[155] blocks NO/cGMP signaling. The priority of TSP1-CD47 interaction in blocking NO-driven events in vascular cells is underscored by the fact that 10 pM TSP1 is sufficient to inhibit NO signaling via CD47, whereas over 100-fold greater concentrations of TSP1 are required to inhibit via CD36.

5.9. Thrombospondin-1 Inhibits NO/cGMP Signaling at Multiple Levels

In murine, porcine and human vascular cells NO-stimulated cGMP accumulation is potently blocked by TSP1/CD47 signaling.[139,153,154] Because the inhibitory activity of TSP1 was preserved in the presence of cGMP PDE inhibitors, sGC must be a direct target of inhibitory TSP1/CD47 signaling (Figure 3). The molecular pathways that transmit this signal from CD47 to sGC remain to be determined.

Remarkably, sGC is not the only target of inhibitory TSP1 signaling. Functional responses of endothelial cells to a cell-permeable analogue of cGMP are also inhibited by TSP1.[139] NO decreases the ability of platelets to aggregate and form thrombi,[156] and TSP1 blocks this effect of NO, promoting platelet adhesion and aggregation.[157] The delay of thrombin-induced platelet aggregation by 8-Br-cGMP was also reversed by TSP1. 8Br-cGMP-stimulated phosphorylation of platelet VASP at Ser^{239} was also inhibited by TSP1. Because this phosphorylation is mediated by cGMP-dependent kinase (cGK), this enzyme appeared to be a second target of TSP1 inhibitory signaling. Regulation of cGK was confirmed using an in vitro kinase assay. 8-Br-cGMP increased Ser phos-

phorylation of the cGK peptide substrate RKRSRAE, and this stimulated phosphorylation was completely blocked by TSP1 or by a specific cGK antagonist.[157]

TSP1 control of physiologic NO signaling also extends to events above sGC activation at the level of endogenous NO production (Figure 3). Zhu et al. reported that extracellular myristate activates eNOS in a CD36- and AMP kinase-dependent manner.[158] Because CD36 is a major transporter of free fatty acids into cells,[159] we proposed that the inhibitory effect of TSP1 mediated by CD36 involves inhibition of its fatty acid translocase activity. Using radiolabeled myristate, we found that TSP1 (and an inhibitory CD36 antibody) block myristate uptake into endothelial cells at concentrations consistent with their activities to inhibit NO/cGMP signaling.[160] This is associated with a block in myristate-stimulated sGC activation and increases in cellular cGMP. Studies of membrane translocation of the Src kinase Fyn showed that extracellular myristate stimulates Fyn translocation and Src kinase activation in a CD36-dependent manner, and TSP1 inhibits these events. Because Src is known to activate eNOS by both direct phosphorylation and phosphorylation of Hsp90, TSP1 may inhibit eNOS activation via this pathway. Alternatively, Zhu et al. showed that the activity of myristate to activate eNOS is AMP kinase-dependent.[158] Therefore, a second inhibitory pathway may be through AMP kinase (Figure 3).

Taken together, these studies show that TSP1 redundantly modulates NO/cGMP signaling in vascular cells at three distinct levels: eNOS activation, sGC activation, and downstream at the level of the cGK (Figure 3). This should enable TSP1 to be a highly effective physiological antagonist of NO signaling. Because tissue or circulating TSP1 levels are elevated in several disease states, this redundant inhibition must be considered in efforts to improve NO signaling. Therapeutic approaches designed to enhance NO signaling at any one of the levels will not bypass inhibition by TSP1 at downstream sites. A more rational approach to maximize the therapeutic potential of physiologic NO would target the necessary receptor CD47.

5.10. TSP1/CD47 Signaling Acutely Regulates Blood Flow and Tissue Survival

Intracellular cGMP, though promoting cell survival in mammalian cells, plays a much more critical acute role in cardiovascular physiology.[161] Through direct modulation of the contractile apparatus of VSMC, cGMP controls blood vessel diameter and, thus, controls blood flow.[162] The key protein in the contractile machinery of VSMC is myosin light chain 2 (MLC2), which activates myosin light chain and enhances Actin-myosin cross-bridge cycling.[163,164] NO stimulates the dephosphorylation of MLC2 and, thus, disrupts Actin−myosin cross-bridge cycling and relaxes VSMC. This then leads to vessel dilation and increased blood flow. However, in the presence of TSP1, NO cannot dephosphorylate MLC2. In vitro TSP1 blocks NO-stimulated relaxation of contracting VSMC.[165] However, treatment of CD47 null VSMC with TSP1 does not block NO-driven relaxation. These in vitro findings translate directly to regulation of blood flow in the whole animal. Mice treated with NO show a predicted increase in tissue blood flow. However, a similar NO challenge in TSP1 (or CD47) null mice results in over twice the increase in regional blood flow compared to wild-type animals. Then endogenous TSP1 is regulating acute blood vessel response to NO.[165] TSP1-CD47 inhibition of NO stimulated vasodilation and NO-driven increases in blood flow is always present and acts as a rheostat upon NO responses in the vasculature limiting in real time the dynamic range of NO effects.

The implications of this discovery are profound. Tissue units exposed to acute ischemic stress via vascular interruption demonstrate enhanced tissue survival and blood flow in the absence of TSP1 or CD47.[166] Conversely, such ischemic stress, in the presence of TSP1-CD47 inhibition of NO signaling, leads to profound loss of tissue blood flow and tissue necrosis. Conversely, blocking TSP1-CD47 signaling with monoclonal antibodies or gene-silencing techniques dramatically enhances ischemic tissue survival and blood flow in both murine and porcine models of acute tissue ischemia.[166] Targeting TSP1-CD47 results in immediate effects upon vascular response to vasoactive stress as documented by laser Doppler or EPR tissue oximetry and parallels the immediate enhanced blood flow enjoyed in null mice exposed to ischemic vasoactive stress in soft tissues and hindlimb models.

In a number of acute and chronic pathologic states, blood flow becomes interrupted and then restored at a later time. This phenomenon is termed ischemia/reperfusion injury (I/R) and is a major source of tissue and organ damage/loss.[167] NO is known to be tissue-protective in I/R.[168] However, administration of NO donors has produced limited therapeutic benefits for I/R injury. We found that TSP1 is rapidly induced following a liver I/R injury, suggesting that TSP1 limits the beneficial activity of NO.[169] Consistent with this hypothesis, null animals lacking TSP1 or CD47 were remarkably resistant to visceral organ I/R injury. Blocking the TSP1-CD47 pathway in wild-type animals also confers dramatic tissue-protective effects to I/R injury.

TSP1−CD47 signaling also limits tissue survival in conditions of complete absence of blood flow, as found in full-thickness skin grafting. In wild-type mice, full-thickness skin grafts experience complete necrosis. In contrast, full-thickness skin grafts in TSP1- and CD47-null animals enjoy essentially 100% survival.[170] Agents that interrupt TSP1−CD47 signaling facilitate the effects of endogenous NO and greatly increase full-thickness skin graft survival in the wild-type animals.

5.11. Can TSP1/CD47 Antagonism of NO Signaling Control Tumor Perfusion?

On the basis of the evidence that TSP1/CD47 signaling acutely limits NO-mediated vasodilation in healthy and ischemic tissues, we examined whether this regulation extends to the tumor vasculature. This is an important question for cancer therapy in that many have sought to acutely increase tumor perfusion to enhance responses to intravenous chemotherapy and to enhance radiation killing to tumor cells by increasing the local oxygen tension.[171] In contrast to normal tissue, systemic administration of NO results in a net decrease in blood flow in the tumor.[172] This is consistent with the known impairment of function in the tumor vasculature.[173,174] Thus, the tumor behaves as a passive resistance to blood flow, and vasodilation of other peripheral tissues by NO decreases flow through the tumor. This is generally known as a "steal effect".[175] When TSP1 was overexpressed by the tumor cells, we found that the magnitude of this decrease diminished, suggesting that TSP1 secreted by the tumor was generally inhibiting the global vascular response to the administered NO. Conversely,

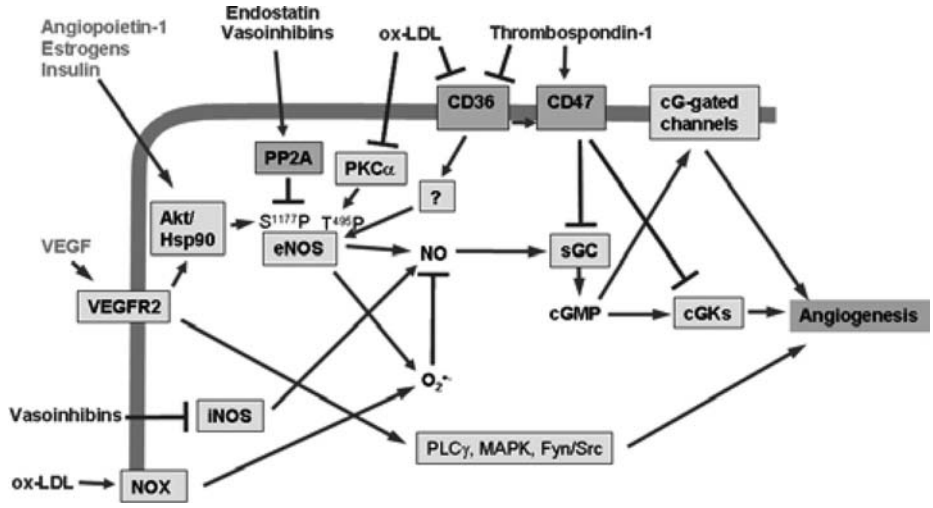

Figure 4. NO/cGMP pathway is a signaling node for pro- and antiangiogenic signaling. In addition to VEGF, angiopoietin-1, estrogens, and insulin can activate eNOS to increase NO synthesis in endothelial cells. TSP1 signaling via CD36 and CD47 inhibits downstream elements of this pathway. Oxidized LDL (oxLDL) is another known ligand of CD36 that could inhibit angiogenesis via an overlapping pathway. In addition, oxLDL signaling inhibits protein kinase Cα, which phosphorylates eNOS at Thr^{495} and activates NADPH oxidase (NOX), and the resulting superoxide depletes NO levels. The angiogenesis inhibitors endostatin and vasoinhibins activate the phosphatase PP2A, which dephosphorylates and thereby inactivates eNOS.

overexpression of TSP1 in the tumor also decreased the net increase in tumor blood flow induced by treating the mice with the vasoconstrictor norepinephrine. These observations may provide an explanation for reports that some murine and human cancers are associated with elevated circulating TSP1.[176,177] The circulating TSP1 may selectively constrict vessel beds outside of the tumor and thereby increase blood flow into the tumor. The growth advantage thus provided would be a selective pressure for maintaining elevated TSP1 expression. Several endogenous angiogenesis inhibitors were first identified based on their elevated circulating levels in tumor-bearing mice, but the reason for their presence was unclear. We propose based on the signaling pathways discussed here that those circulating inhibitors may have similar acute effects to enhance tumor perfusion.

5.12. TSP1/CD47 Antagonism of NO Signaling Controls Tissue Radiosensitivity

NO donors have known radioprotective activities for whole-body irradiation of mice[178] and for gamma or UV irradiation of cells in vitro.[179,180] This suggested that ablating TSP1/CD47 signaling could protect tissue from radiation injury by enhancing prosurvival NO signaling. This hypothesis was tested by irradiating the hindlimbs of wild type, TSP1 null, and CD47 null mice.[181] Remarkably, at 25 Gy irradiation, both null mice were essentially protected from the effects of irradiation. Hair loss (alopecia) was absent in CD47 null and decreased in TSP1 null mice. Both null mice showed minimal apoptosis in skeletal muscle and bone marrow 24 h following irradiation, and muscle function was preserved 2 months following irradiation. Remarkably, radioprotection in the null mice extended to isolated cultures of vascular cells. These cells survived irradiation at up to 40 Gy and remained competent to replicate their DNA postirradiation. This identifies the TSP1/CD47 pathway as a limiting pathway for recovery from radiation injury and suggests that targeting this pathway could protect adjacent healthy tissue from radiation injury due to radiotherapy of tumors or following accidental or military-related exposure to radiation.

5.13. Do Other Angiogenesis Inhibitors Block NO Signaling?

TSP1 may be unique in its redundant regulation of the NO/cGMP signaling cascade, but increasing evidence indicates that the same pathway is a target of additional endogenous angiogenesis inhibitors (Figure 4). Prolactin-derived vasoinhibins were shown to inactivate eNOS via protein phosphatase 2A.[182,183] A second study indicated that vasoinhibins can further limit NO signaling through down regulation of iNOS.[184]

NO signaling has also been identified as a target for the antiangiogenic activity of endostatin. Endostatin reduces VEGF-induced phosphorylation of eNOS at Ser^{1177}, independent of any change in Akt phosphorylation.[185] This was attributed to activation of PP2A, which dephosphorylates eNOS at Ser^{1177}. Furthermore, sGC protein levels were suppressed following treatment with endostatin.[186] The decrease in sGC protein was not associated with a decrease in mRNA levels, indicating that regulation is post-transcriptional. PP2A was also implicated in this response based on abrogation in the presence of okadaic acid.

Oxidized LDL was also shown to inhibit VEGF-induced endothelial cell migration by blocking Akt-mediated phosphorylation of eNOS at Ser^{1177} and thereby decreasing NO production.[187] Subsequent studies showed that oxidized LDL also decreases the phosphorylation of eNOS on Thr^{495} via PKCα, and this was accompanied by increased $O_2^{\bullet -}$ production due to uncoupling of eNOS.[188] Furthermore, oxidized LDL increases NADPH oxidase activity in endothelial cells, which further increases $O_2^{\bullet -}$ production.[189] This increased $O_2^{\bullet -}$ could further lower cGMP signaling by consuming available NO.[190] Like TSP1, oxidized LDL is a ligand for CD36,[191] and TSP1 is known to inhibit eNOS activation by blocking myristate uptake via CD36.[160] However, whether CD36 is the receptor that mediates the above activities of oxidized LDL has not been established.

6. Hydrogen Peroxide and Angiogenesis

6.1. H_2O_2 as a Signaling Molecule

Reactive oxygen species (ROS) have been negatively associated with many different aspects of cardiovascular disease such as hypertension, atherosclerosis, heart failure, and restenosis.[192] Recently, however, ROS, and particularly hydrogen peroxide, have been recast as important second messenger molecules that respond to a variety of cytokines and growth factors. Examples include tumor necrosis factor α (TNFα),[193] platelet-derived growth factor (PDGF),[194] epidermal growth factor (EGF),[195] and insulin,[196] all of which elicit a transient increase in H_2O_2. A review of all of the essential roles of H_2O_2 signaling is outside the scope of this text but is available elsewhere.[197]

In the context of tumor angiogenesis, the recognized roles of H_2O_2 in signaling are rapidly expanding and are the subject of active research. Importantly, ROS such as $O_2^{\bullet -}$ and H_2O_2 are increased in numerous cancer cells.[198] Exogenously added as well as cellularly derived H_2O_2 stimulates angiogenic responses in cultured endothelial cells and smooth muscle cells as well as in tissue and animal models of angiogenesis (reviewed in ref 199). Brauchle et al. first documented the ability of H_2O_2 to directly stimulate the production of VEGF from cultured keratinocytes while investigating the effects of UV irradiation.[200] The connection between cellular ROS/H_2O_2 levels and VEGF production was made at about the same time based on both signals being elevated following I/R injury.[201] H_2O_2 stimulates the production of VEGF-A protein and mRNA in a variety of cell types including rat VSMC,[202] rat heart endothelial cells,[203] C2C12 skeletal myotubes,[204] human and rat macrophages,[205] NIH 3T3 cells,[206] and DU-145 prostate carcinoma cells.[206] The addition of antioxidants such as *N*-acetylcysteine and green tea catechins to scavenge $O_2^{\bullet -}$ and H_2O_2 has been shown to inhibit angiogenesis in vivo.[207]

In addition to VEGF signaling, stromal cell-derived factor 1 (SDF-1) receptor CXCR4 mRNA expression is upregulated maximally in the presence of 10 μM H_2O_2.[208] Consistent with this, H_2O_2 plays a critical role in the mobilization, homing, and angiogenic capacity of bone marrow-derived endothelial progenitor cells (EPC).[209] To critically assess the putative and as yet undefined targets of H_2O_2 in angiogenic signaling, one must first understand the basic chemical biology of H_2O_2.

H_2O_2 is the product of biological reduction of dioxygen. Most H_2O_2 in cells derives from the dismutation of superoxide anion ($O_2^{\bullet -}$). $O_2^{\bullet -}$ can be generated by several enzymes including xanthine oxidase, cytochrome p450s, uncoupled NOS, and myeloperoxidase, although the main sources of cellular $O_2^{\bullet -}$ for the production of H_2O_2 are NADPH oxidases (NOXs)[210] and the mitochondria through electron transport chain-associated enzymes.[211,212] From here, $O_2^{\bullet -}$ can be converted to OONO− by reaction with NO, to hydroxyl radical (HO•) by Fenton or Haber−Weiss processes, or to H_2O_2 by superoxide dismutase. Alternatively, H_2O_2 can be formed directly from dioxygen by DuOXs (dual-function oxidases)[210] or oxidoreductases such as glucose oxidase.[213]

6.2. NADPH Oxidase in Endothelial Cells

The main source of $O_2^{\bullet -}$ in endothelial cells is the NADPH oxidase system.[214] NADPH oxidase was first characterized in phagocytic cells (neutrophils) and is a complex enzyme composed of five different regulatory subunits. A membrane spanning cytochrome b558 is composed of gp91phox (Nox2) and p22phox as well as the cytosolic components p47phox, p67phox, and the small GTPase Rac.[215,216] NOX2 contains an NADPH binding site on the cytosolic portion of the protein as well as a flavin adenine dinucleotide (FAD) binding site facilitating electron transfer.[216] Gp91phox uses pairs of histidine residues to bind two hemes in a hexacoordinate low-spin fashion, whereby the outer heme can reduce O_2 to $O_2^{\bullet -}$ through rapid outer-sphere electron transfer when reducing equivalents are available from NADPH:[217,218]

$$O_2 + His_2Fe^{2+}PPIX \rightarrow O_2^{\bullet -} + His_2Fe^{3+}PPIX \quad (1)$$

This also implies that the hemes of NADPH oxidase cannot be "poisoned" by CO or CN^-, though there is one report that CO can interact with the cytochrome b558 heme of Nox2 under nonphysiological conditions.[219] The cytosolic components are necessary to activate the electron transfer. They facilitate catalysis by translocating to the membrane, in a Rac1- or Rac2-dependent manner, assembling with the plasma membrane subunits.[216,220] Vascular cell and phagocytic cell NOX differ in the manner in which they produce $O_2^{\bullet -}$. Neutrophil NOX2 produces large concentrations of $O_2^{\bullet -}$ in short bursts, while vascular NOXs produce a sustained low level of $O_2^{\bullet -}$ that can be enhanced acutely by growth factors and other cell ligands.[221] Several paralogs of gp91phox (Nox2) that share 30−60% sequence homology are expressed in vascular cells as part of the NOX complex including Nox1, Nox4, and Nox5.[222,223] Each shares the common NADPH, FAD, and heme binding sites. Endothelial cells also express all of the other canonical NOX subunits with similar regulation.

$O_2^{\bullet -}$ from NADPH oxidase is produced on the opposite side of a plasma membrane from the electron source (NADPH). This may be into the extracellular space or into a subcellular membrane compartment ranging in size from a group of signaling proteins or containing a major organelle. Unlike other small-molecule signaling agents (NO, CO, O_2), $O_2^{\bullet -}$ is anionic and cannot freely diffuse across membranes. $O_2^{\bullet -}$ is then restricted as a signal by being able to move across membranes only with the aid of ion channels or after reduction to neutral diffusible H_2O_2. $O_2^{\bullet -}$ is converted into H_2O_2 by a group of proteins known as superoxide dismutases:

$$2H^+ + 2O_2^{\bullet -} + SOD \rightarrow H_2O_2 + O_2 + SOD \quad (2)$$

Mammals express three isoforms: a cytosolic CuZnSOD (SOD1), a mitochondrial MnSOD (SOD2), and an extracellular CuZnSOD (SOD3 or ecSOD).[224] Although the dismutation of $O_2^{\bullet -}$ is spontaneous, these enzymes catalyze the process at rates approaching 10^9 M^{-1} s^{-1}. While the other two isoforms are ubiquitous, ecSOD is the major isoform found in the vascular extracellular space secreted by endothelial cells, smooth muscle cells, and fibroblasts.[224] It binds to the extracellular matrix near endothelial cells through interactions with collagens, heparan sulfate proteoglycans, and fibulin-5. The subcellular localization of NADPH oxidase determines the relevance of each SOD isoform. SOD1 is the most relevant to signaling in intracellular vesicles, while ecSOD handles $O_2^{\bullet -}$ produced in the extracellular space.

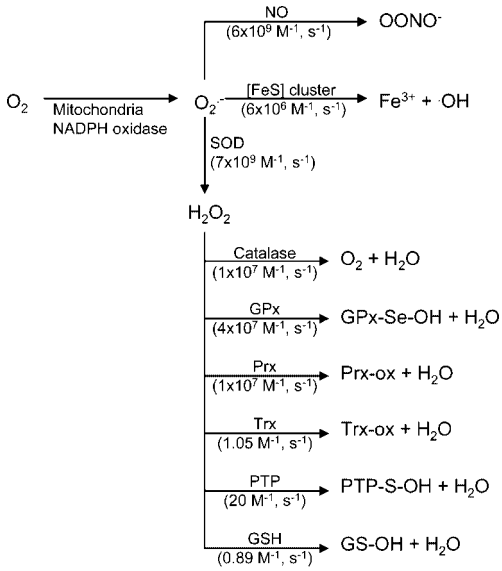

Figure 5. Superoxide and hydrogen peroxide reactivity. Superoxide generated by the mitochondria and NADPH oxidase has three main cellular fates: reaction with NO to form superoxide, reaction with iron−sulfur cluster (Fe−S) proteins to release ferric iron and hydroxyl radical, and reaction with superoxide dismutases (SOD) to form hydrogen peroxide. Hydrogen peroxide is consumed in scavenging and signaling reactions over a wide range of second-order rate constants (references: NO,[411] [Fe−S],[412] SOD,[413] catalase,[225,414] GPx,[415] Prx,[416] Trx,[417] PTP,[418] and GSH[419]).

6.3. H_2O_2 Targets in Vascular Cells

H_2O_2 is consumed by a number of different enzyme systems. Catalase is a heme protein that uses one molecule of H_2O_2 as an oxidant to oxidize a second equivalent of H_2O_2, generating water and molecular oxygen:

$$H_2O_2 + Cat{-}Fe^{3+} \rightarrow H_2O + Cat{-}Fe^{4+}{=}O \quad (3)$$

$$H_2O_2 + Cat{-}Fe^{4+}{=}O \rightarrow O_2 + H_2O + Cat{-}Fe^{3+} \quad (4)$$

Catalase reacts with H_2O_2 with a second-order rate constant of 10^7 M^{-1} s^{-1} in order to maintain the level of H_2O_2 and minimize any promiscuous oxidations.[225] In addition to catalase, H_2O_2 is consumed by cellular thiols and is a major contributor to the thiol redox status of the cell. The thiol redox status is controlled by two major systems, the thioredoxin system and the glutathione system. H_2O_2 interacts with these two systems by reactions with peroxiredoxins (Prx) and glutathione peroxidases (Figure 5). Prx are dithiol-containing enzymes that are converted to the disulfide by H_2O_2. Prx are reduced by thioredoxin, which is in turn reduced by the selenocysteine- and NADPH-dependent thioredoxin reductase. Glutathione peroxidase, on the other hand, uses selenocysteine to reduce H_2O_2, generating a selenic acid that is recycled using glutathione, which is in turn reduced by glutathione reductase in an NADPH-dependent fashion.

Early studies using rat livers estimated the intracellular H_2O_2 concentration to range from 0.001 to 0.1 μM.[226,227] The maximum proliferative level was determined to be 0.7 μM in Jurkat T-cells, above which apoptosis occurs.[228] It is important to note that there is a concentration gradient from the outside to the inside of the cell when administering H_2O_2 experimentally due to the membrane permeability and intracellular consumption of H_2O_2. Thus, the intracellular concentration is approximately 7- to 10-fold less that the extracellular concentration.[229] Accordingly, stimulated rat brain extracellular H_2O_2 levels were measured amperometrically to be 2−4 μM, which would correspond to 0.2−0.4 μM inside the cell, well under the upper physiological limit of 0.7 μM.[230]

The signaling properties of H_2O_2 derive mostly from its electrophilic character and ability to react with protein thiols, oxidizing them to sulfenic and then to sulfinic acids:[231]

$$RSH + H_2O_2 \rightarrow RSOH + H_2O \quad (5)$$

$$RSOH + H_2O_2 \rightarrow RSO_2H + H_2O \quad (6)$$

H_2O_2 reacts with thiols (RSH) relatively slowly, but the reaction rate is enhanced by deprotonation of the thiol to a thiolate anion (RS^-) with a modest rate of 10−100 M^{-1} s^{-1}. For H_2O_2-based signal transduction to occur, some thiolate proteins have evolved with an active site pocket that significantly lowers the transition state energy of the H_2O_2−thiolate reaction such that the reaction rate can be as fast as 10^5 M^{-1} s^{-1}.[232] The thiolate-containing proteins having the fastest rate constant for H_2O_2 of course are the peroxidases, but the body contains other important thiolate-dependent proteins whose primary role is not the degradation of peroxides such as phosphatases, thiol−disulfide isomerases, glutathione S-transferases, dehydrogenases, and transglutaminases.[233] These proteins react with H_2O_2 at rates 3−5 orders of magnitude slower that the peroxidases[197] and are not expected to interact with H_2O_2 below the 0.2−0.4 μM levels described in stimulated cells described above (vide supra). Thus, it is more likely that physiological H_2O_2 signaling occurs in "bursts" that produce in excess of 0.2 μM but less than 0.7 μM H_2O_2.

6.4. H_2O_2 Regulation of Vascular Tyr Kinase Signaling

One important established role of peroxide signaling is the potentiation of receptor tyrosine kinase signaling by oxidation of protein tyrosine phosphatases (PTP) that negatively regulate their signaling.[234] A majority of PTPs rely on a cysteine thiolate in their active site for their phosphatase actions.[235] Reversible oxidation of this moiety by H_2O_2 can shift the equilibrium in favor of enhanced tyrosine phosphorylation and downstream signaling (Figure 6). The result is to extend the duration of cellular responses following ligation of Tyr kinase receptors.

As discussed above, VEGFR2 is a transmembrane receptor activated by ligand stimulated dimerization and trans(auto-)phosphorylation of cytoplasmic facing tyrosine residues ($Tyr^{951,996,1054,1059}$).[236] H_2O_2 is implicated as an important mediator of VEGF angiogenic signaling in endothelial cells through VEGFR2 phosphorylation and enhanced phosphorylation of downstream targets such as c-Src and VE-cadherin.[237−239] Ushio-Fukai et al. have shown also that VEGF-induced VEGFR2 autophosphorylation is inhibited by the H_2O_2 scavenger *N*-acetylcysteine, NADPH oxidase inhibitors, and either gp91phox antisense oligonucleotides or overexpression of a dominant negative form of Rac1.[237] Potentiation of receptor tyrosine kinase signals is common for other angiogenic factors as well. As mentioned above, angiopoietin-1 signals through a receptor tyrosine kinase known as Tie2, and its effects are inhibited by overexpression of catalase.[240,241] Angiotensin II induces H_2O_2-dependent phosphorylation of epidermal growth factor receptor (EGFR) tyrosine residues.[242] Additionally, insulin

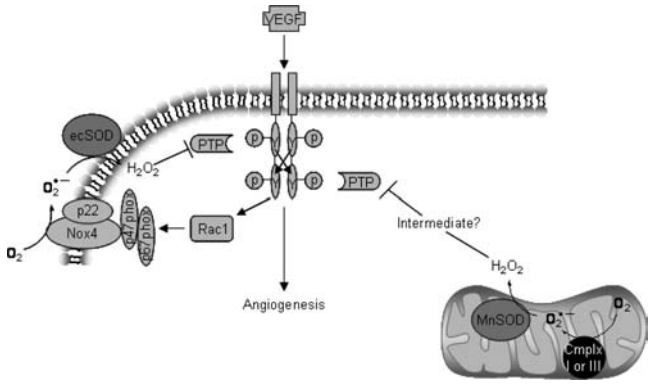

Figure 6. VEGF signaling leads to H_2O_2 production and positive feedback through inhibition of PTPs. VEGF binding to its receptor (VEGFR) results in autophosphorylation of tyrosine residues, leading to downstream kinase activation and angiogenic activity. VEGFR activation also leads to activation of the small GTPase RAC1, which, in conjunction with other cytosolic components (gp47- and p67phox), activates NADPH oxidase. NADPH oxidase reduces dioxygen to superoxide subsequently converted to H_2O_2 by SODs. H_2O_2 either directly or through a thiol peroxidase intermediate inactivates PTPs that oppose the actions of VEGFR, thus enhancing and sustaining the downstream signal. Another source of superoxide is from the reduction of oxygen by the uncoupling of the mitochondrial electron transport chain (through complex-I or -III). This may contribute to the potentiation of VEGFR signaling as well.

and PDGF-dependent autophosphorylation of their respective receptors is inhibitable with catalase overexpression.[194,243]

The targets of this signaling are specific PTPs. In the case of VEGFR2, SHP-1, SHP-2, and HCPTPA are known to associate with activated VEGFR2. Reversible redox regulation has been reported for each of these PTPs.[244−246] For example, SHP-2 oxidation by H_2O_2 in vitro resulted in active site cysteine oxidation and decreased PTP activity.[247] Importantly, the sensitivity to oxidation was greater when the protein was without its SH2 domain, which it uses to dock with VEGFR. Under basal (nonsignaling) conditions, the SH2 is folded over to protect the catalytic domain from oxidative inactivation. Thus, VEGF-stimulated production of H_2O_2 constitutes a positive feedback loop that potentiates the actions of it and other similar receptor tyrosine kinase dependent growth factors (Figure 6).

6.5. H_2O_2 Regulation of Vascular Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are essential to many different facets of angiogenesis. MMPs actively maintain the balance between pro- and antiangiogenic factors by generating matrix protein fragments in both categories (Table 2) and clearing cytokines from membrane-bound states while also using their proteolytic ability to degrade endothelial basement membrane to facilitate cell migration and providing a proper space for endothelial cell lumen development. MMPs belong to a family of zinc-containing endopeptidases, of which at least 28 different isoforms have been identified in humans.[248] MMPs can be secreted or membrane bound (MT-MMPs). The membrane-bound class includes four transmembrane MMPs (MT1-, MT2-, MT3-, and MT5-MMP) and two that are glycosylphosphatidylinositol anchored (MT4- and MT6-MMP). As a group, these MMPs can degrade all recognized mammalian extracellular matrix proteins,[249,250] while MMP-2, MMP-9, and MT1-MMP are critical to angiogenesis.[251,252] In addition to MMPs, several metalloproteinase homologues perform important pericellular angiogenic functions. These are known as ADAMs (A Disintegrin and Metalloproteinase Domain) and ADAMTs (A Disintegrin and Metalloproteinase and Thrombospondin motifs).

The MMPs are regulated at multiple levels including gene expression, compartmentalization (pericellular), zymogen activation, and enzyme inactivation. MMPs are not produced in an active state but are rather in a pro-state (zymogen) waiting for an angiogenic signal in the form of wound repair, inflammation, or tumor perfusion to induce their activation. This is due to the structure of MMPs containing a built-in autoinhibitory pro-domain as well as a catalytic domain. The autoinhibitory domain has a critical cysteine residue that coordinates the catalytic site $Zn^{2+,}$ thereby blocking coordination of other peptides. For the enzyme to be active, the thiol$-Zn^{2+}$ interaction must be disrupted. This is known as a cysteine switch as first proposed by Van Wart et al. The switch can happen in three basic ways: (i) proteolytic cleavage of the autoinhibitory domain by another protease (serine protease or MMP); (ii) oxidation of the inhibitory cysteine thiol by ROS, heavy metals, or thiol modifiers; and (iii) allosteric modification of the zymogen resulting in intramolecular proteolytic cleavage of the autoinhibitory domain. H_2O_2 has the potential to activate at least two of these pathways. Direct oxidation of the inhibitory Cys residue by H_2O_2 is suggested in work by Grote et al. in which mechanical stress-induced MMP activation was highly NADPH oxidase dependent[253] as well as work citing the inhibitory effects of antioxidants on the activation of MMPs.[254] H_2O_2 can also stimulate the proteolytic activation of MMPs by inducing expression of proteinases such as urokinase plasminogen activator and MMP-1 through Ets-1.[255] Furthermore, H_2O_2 stimulation leads to enhanced expression of MMPs through a mechanism that is dependent on NADPH oxidase activity.

6.6. Intracellular Vascular Targets of H_2O_2

H_2O_2 is also a direct effector of angiogenic signaling downstream of cell surface receptors via redox-sensitive H_2O_2-dependent activation of transcription factors. These generally have a regulatory cysteine residue that, when oxidized, modulates the transcriptional activity of the protein. Examples of these relevant to angiogenesis are NF-κB,[256,257] Ets-1,[258] and p53.[259]

The reaction of H_2O_2 with any of the above-mentioned signaling proteins is 3−5 orders of magnitude slower than with the H_2O_2 scavenging proteins (Figure 5). For efficient signaling to occur, the H_2O_2 concentration would have to significantly increase (say, 3−5 orders of magnitude) or the concentration of scavenging proteins would have to be decreased. Alternatively, a recent review by Ushio-Fukai explains that location may be critical for NADPH oxidase signaling, thereby addressing some of the issues of H_2O_2 concentrations and scavengers.[260] During angiogenesis, endothelial cells become polarized, and migration is directed by formation of lamellipodia and filopodia at the leading edge. Migration also depends on spatially and temporally restricted localization of signaling molecules including Rac1 (NADPH oxidase generated $O_2^{\bullet-}$) and PI3K (PIP3). Accordingly, NADPH oxidase is localized to signaling complexes at the leading edges of migrating cells, which could spatially modulate the activities of the lipid phosphatase PTEN, PTPs, and receptors such as EGFR.[260] Another

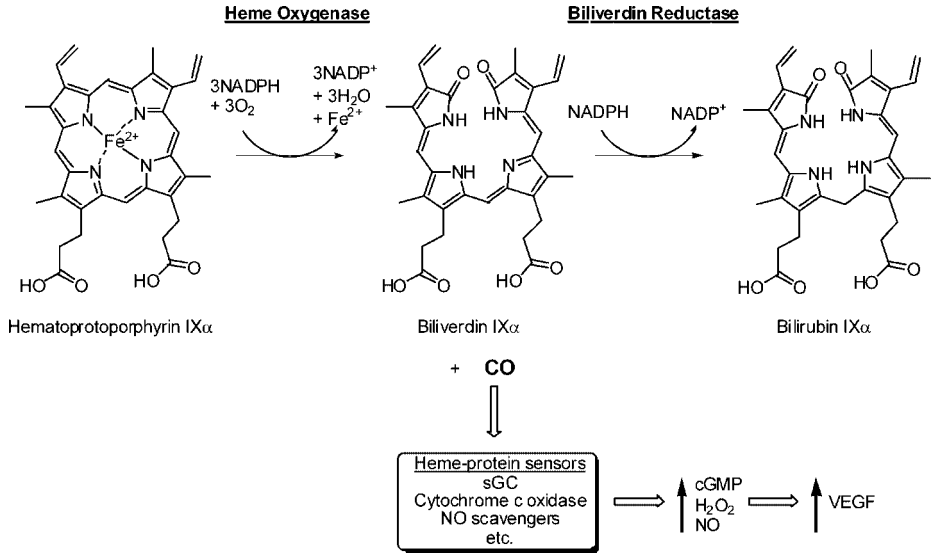

Figure 7. CO biosynthesis. Carbon monoxide is synthesized by the heme oxygenases (HOs). HOs bind heme and use it to activate oxygen to catalyze its own oxidation to biliverdin and CO. Biliverdin is subsequently reduced by biliverdin reductase to bilirubin. CO signaling is exclusively due to interaction with reduced heme proteins including those that are targets of NO and O_2.

perspective on the mechanism of H_2O_2 signaling involves the intermediacy of other oxidized thiol protein such as Prx, thioredoxins, or glutathione (see Figure 5) to mediate the signal. An example of this is the reaction of oxidized yeast GPx3 with the transcription factor Yap1.[261] Oxidation of GPx3 produces sulfenic acid, which then reacts with a Yap1 cysteine residue, forming an intermolecular disulfide that then resolves into an intramolecular disulfide and activates the signal. In this way, Prx acts as the local sensor of H_2O_2 and extends the lifetime of the signal to find the specific target protein thiol. This suggests that Prx and the like have two functions: scavenging H_2O_2 and propagating its signal. Proponents of this dual role often refer to a floodgate hypothesis governing where Prx consume low levels of H_2O_2 and act as signals in the presence of higher concentrations that could overoxidize Prx to the sulfinic acid.[262,263]

Although it is clear that H_2O_2 plays a significant role in angiogenic signaling, more work needs to be done to address the specific regulation of NADPH oxidases by angiogenic regulators and to clarify how specificity in regulating downstream targets is achieved.

7. What is the Contribution of Carbon Monoxide to Angiogenesis?

Until NO became widely recognized as a key endogenously generated signaling molecule, CO was regarded as an uninteresting byproduct of heme degradation. However, recent work suggests that endogenously generated CO also plays a significant physiological role. Numerous cardiovascular and immunological effects have been reported for both endogenous CO and pharmacological CO donors. Unlike NO, however, no specific CO target has been identified that clearly mediates its effects. In the context of angiogenesis, CO may derive some of its effects as a discrete signaling moiety but also (maybe more importantly) via its effects on NO and O_2 signaling.

7.1. Introduction to Carbon Monoxide Chemistry and Physiology

CO is a colorless gas at room temperature and pressure. Like NO and O_2, it favorably partitions into hydrophobic membranes and has a similar water solubility (2.5 mM). However, unlike NO and O_2, the physiological chemistry of CO is relatively simple. It is unreactive toward the other diatomic gases and most biological molecules. CO can be oxidized to CO_2 by the mitochondria,[264] but this is not a major path for CO elimination, which is primarily via exhalation through the lungs.[265] The most significant aspect of biological CO chemistry is its ability to bind to transition metals in metalloproteins, particularly reduced heme proteins (as reviewed in ref 266). CO competes with NO and O_2 for binding to some heme proteins, thus affecting their signaling pathways. However, CO binds to these heme proteins in a different manner than O_2 and NO. The binding geometry for NO can be bent or linear depending on the electronics of the overall complex, and O_2 prefers a bent geometry. CO, on the other hand, binds only in a linear mode. This becomes important when considering the relative affects of CO, NO, and O_2 signaling through proteins capable of interacting with all three (such as sGC).

CO interacts with several heme proteins that regulate important signaling pathways as reviewed by Wu in 2005.[267] The one known example of CO being an agonist for these targets is the NO binding protein sGC, where CO is an activator, albeit much weaker than NO. The affinity constants are 2.6×10^{-4} for CO[125] and 4.2×10^{-12} for NO.[268,269] CO can elicit cardiovascular effects such as vasorelaxation and inhibit platelet aggregation by directly activating sGC.[270] Because of this competition, periods of hypoxia or oxidative stress can modulate CO binding to heme protein signaling pathways. However, CO is unlikely to be a significant antagonist of NO for sGC activation because its binding to sGC is too weak.

7.2. Biogenesis of CO

Biological generation of CO is largely due to the enzymatic degradation of heme by heme oxygenase enzymes (Figure 7). These enzymes catalyze the primary and rate-limiting step in heme catabolism using NADPH and dioxygen to produce 1 equiv of CO, biliverdin-IXα, and Fe^{2+} per equiv of heme.[271] Mammalian cells express two isoforms of heme oxygenase: a highly inducible (HO-1) 32 kDa isoform and

a noniducible (HO-2) 36 kDa isoform. A third isoform (HO-3) has recently been cloned from rat brain cDNA, but it is much less active than HO-1 or HO-2. For reviews on heme oxygenases, see refs 267, 270, 272, and 273. HO-2 is constitutively found in the brain, intestines, testis, and endothelium. HO-1 is responsible for metabolizing the heme released during red blood cell turnover, and as a result, it is constitutively found in the spleen and liver. However, HO-1 can also be induced in most tissues in response to a diverse set of stress signals. These include ROS, UV irradiation, heat shock (HO-1's alternate name is HSP32), hypoxia, hyperoxia, heavy metals, NO, inflammatory cytokines, heme, and ethanol.[273] The common feature shared by all these stimuli seems to be that they induce oxidative cell stress. Therefore, HO-1 induction is thought to be part of the antioxidant response. Indeed, HO-1 induction protects against a number of pathophysiological insults including myocardial reperfusion toxicity,[274] organ transplant rejection,[274] hypoxia-induced pulmonary hypertension,[275] and TNFα-mediated endothelial cell apoptosis.[276] It is important to note that, although the coproducts of HO-1 (biliverdin, bilirubin, and Fe^{2+}) are bioactive themselves, most actions of HO-1 in these stress models can be recapitulated by CO.

7.3. CO as a Stimulator of Angiogenesis

In addition to its antioxidant, anti-inflammatory, antiapoptotic, and vasorelaxant effects, HO-1 and its product CO have recently gained interest as mediators of angiogenesis, nowhere more importantly than in the area of tumor biology. Notably, HO-1 expression is increased in a number of human solid tumors including renal cell[277] and prostate carcinoma[278] as well as some experimental solid tumors such as rat hepatoma AH136B[279] and mouse Sarcoma 180.[280] Overexpression of HO-1 in a mouse pancreatic cancer model correlated with significant increases in tumor size, angiogenesis, lung metastasis, and mortality.[281] Correspondingly, the first suggested link between HO-1 and angiogenesis came from correlating HO-1 overexpression with enhanced endothelial cell proliferation.[282] Likewise, endothelial cells made HO-1 deficient using an antisense oligonucleotide exhibited decreased proliferation, in vitro capillary formation, and cell cycle progression, which could be rescued using a CO donor.[283] The effect of the CO donor reinforces the view that the HO-1 product relevant to angiogenesis is CO.

HO-1/CO's influence on angiogenesis is not limited to vascular cells. In vivo studies of human gliomas have linked enhanced HO-1 expression in tumor associated macrophages with greater small-vessel density.[284] This finding is supported by work showing increased expression levels of HO-1 in macrophages associated with melanomas but not in the associated melanocytes, fibroblasts, or keratinocytes.[285] The cell-type-specific expression may be an important caveat when considering HO-1/CO mediated tumor angiogenesis. The in vivo aspects of HO-1-induced angiogenesis were first demonstrated by infusion of an adenoviral vector expressing HO-1 into the femoral artery of a rat, resulting in a significant increase in blood flow and vessel density.[286] These effects could be inhibited using the HO-1 inhibitor Zn-protoporphyrin.[286]

7.4. HO-1 Up-regulation Increases Expression of Angiogenic Factors

As discussed above, VEGF-A is a potent stimulator of angiogenesis through increasing endothelial cell mitogenesis and migration. A growing number of recent studies provide links between VEGF expression and activities of HO-1. In this way, CO may be beneficial to the growth of tumors and play a role in the angiogenic switch for tumor progression.

Inhibition of HO-1 using SnPPIX completely inhibited the release of VEGF from rat VSMC stimulated with IL-1β and hypoxia, whereas NO synthase inhibitors failed to completely inhibit VEGF release.[287] VEGF production was increased by hemin (a stimulator of HO-1 activity) as well as by HO-1 overexpression.[287] Inducers of HO-1 (endotoxin, H_2O_2, and a metalloproteinase) all led to increased VEGF production from rat lung microvascular endothelial cells transfected with an HO-1 expression plasmid.[288] Of the products of HO-1 catalysis, CO (1% atmosphere) was the only moiety able to recapitulate the increase in VEGF seen with HO-1 stimulation or overexpression.[287] Likewise in macrophages, SnPPIX and ZnPPIX significantly decreased the production of VEGF by RAW264.7 cells unstimulated or stimulated with lipopolysaccharide.[289,290]

15-deoxy-Delta(12,14)prostaglandin-J_2 (15d-PGJ_2) is the most potent endogenous ligand for PPARγ activation and is known to up-regulate VEGF expression in VSMC,[290,291] coronary endothelial cells,[292] and macrophages.[290,293] When PPARγ binds a stimulator such as 15d-PGJ_2 or polyunsaturated fatty acid metabolites, it dimerizes with retinoid X receptor, and this complex binds to DNA at specific elements known as PPAR response elements.[294] HO-1 is under the control of this element,[295,296] and induction of HO-1 in turn stimulates VEGF production. In contrast, 15d-PGJ_2 can also stimulate HO-1 expression via the antioxidant response element (ARE) in a PPARγ-independent manner.[297−300] Interestingly, 15d-PGJ_2 is part of a larger group of cyclopentenone prostanoids that are capable of Michael addition chemistry to nucleophilic thiols such as those reported in Ras,[301] leaving open the possibility of direct 15d-PGJ_2 interaction with KEAP/Nrf2. Either way that the HO-1 stimulation occurs, 15d-PGJ_2 stimulation of HO-1 leads to VEGF production, and HO-1 inhibitors (SnPPIX) prevent 15d-PGJ_2 induced VEGF production.[302,303] This is suggested to occur through the phosphorylation of ERK1/2[303] downstream of 15d-PGJ_2 induction of HO-1.

VEGF expression in human umbilical vein endothelial cells (HUVEC) is also stimulated by HO-1 activity as shown with overexpression and chemical stimulation.[289] The ability of an NO donor (SNAP) to stimulate VEGF production in HUVEC was also dependent on the activity of HO-1.[304] High glucose impairs the ability of cells to produce VEGF and is thought to contribute to the decrease in wound healing observed in diabetic patients.[305] Expression of HO-1 restores VEGF levels in keratinocytes cultured under hyperglycemic conditions.[306] VEGF may not be the only angiogenic factor regulated by CO because HO-1 induction also stimulates the release of the angiogenic cytokine IL-8 from human microvascular endothelial cells.[307]

7.5. Stimulators of HO-1 and CO Production Leading to VEGF

Endothelial cells given a positive angiogenic signal (prolactin) increase HO-1 expression associated with endothelial

cell proliferation and angiogenesis.[308,309] A possible feedback system exists whereby VEGF can stimulate the expression of HO-1 to reinforce the angiogenic response. This is suggested in work by Fernandez and Bonkovsky where they observed increased HO-1 expression consistent with angiogenesis 48 h poststimulation of chicken embryo chorioallantoic membranes with VEGF.[310] The effect was inhibited by ZnPPIX. Interestingly, VEGF-driven HO-1 expression was Ca^{2+}-dependent and inhibitable using a PKC inhibitor (staurosporine).[310] This is consistent with the previously known Ca^{2+}/PKC dependence of TNFα and IL-1 induced HO-1 expression,[311] although staurosporine is not a specific PKC inhibitor.

NO has an established role as a mediator of angiogenesis.[312] NO triggers prosurvival and proangiogenic activities in the endothelium by promoting growth and differentiation by activation of the endothelial isoform of NO synthase (eNOS) and signaling through sGC to elevate cGMP levels (see section 4). However, in other cell types such as smooth muscle, tumor cells, keratinocytes, macrophages, and mesangial cells, expression of the inducible form of NO synthase (iNOS) led to HO-1-dependent production of VEGF.[313] NO and CO have a complicated relationship in vivo because they are known to regulate the production of one another (reviewed in refs 314 and 315). Low (nM) concentrations of NO consistent with proangiogenic signaling do not induce expression of HO-1 and appear to inhibit constitutively expressed HO-2.[316] However, higher NO fluxes such as those produced from iNOS,[317] rapidly decomposing NO donors,[318] or organic nitrites[319] are known to induce HO-1 expression and thereby increase the cellular levels of CO. Overexpression of iNOS in smooth muscle cells led to an enhancement of IL-1β-induced VEGF expression that was dependent on HO-1 activity.[320] Treatment of endothelial cells with an NO donor (SNAP) increased the synthesis of VEGF in an HO-1 dependent manner.[304]

7.6. HO-1 and CO Mediate the Proangiogenic Actions of SDF-1

The proangiogenic effects of SDF-1 (aka CXCL12) and its receptor CXCR4 have recently been linked to HO-1 and CO.[321,322] SDF-1 is a major chemokine responsible for the mobilization and targeting of endothelial progenitor cells to sites of injury and new vessel formation. Bone marrow-derived vascular stem cells will migrate to areas presenting a high concentration of SDF-1 such as nascent wounds or tumors.[323] In fact, inactivation of SDF-1 or CXCR4 in mice is embryo lethal due to impaired vascular development.[324,325] SDF-1/CXCR4 have also been implicated in the targeting of metastatic breast cancer cells to the brain[326] and the impairment of EPC recruitment in coronary artery disease and vascular pathology of diabetes.[327] Human endothelial cells as well as EPC exposed to SDF-1 up-regulate HO-1 mRNA, and inhibition of HO-1 impairs SDF-1 driven in vitro angiogenesis.[321] A portion of the angiogenic effect of SDF-1 is through stimulation of VEGF, which, in contrast to the examples cited above, appears to be independent of HO-1.[321] The HO-1-dependent angiogenic actions of SDF-1 can be mimicked with the addition of CO donors.[321] The authors attribute the effects of CO to its well-known activation of sGC and subsequent activation of the cytoskeletal-associated cGK target protein, VASP.[321]

7.7. Decreased CO Contributes to Angiogenesis Inhibition

Dysregulation of angiogenesis is a hallmark of preeclampsia, and HO-1/CO have been linked to successful pregnancy in human and animal models.[328] The antiangiogenic proteins soluble Flt-1 (aka soluble VEGFR-1, Table 2) and soluble endoglin are elevated in preeclamptic women 2−3 months prior to the clinical manifestation of the disease.[329] In pregnant rats, overexpression of soluble Flt-1 induced symptoms mimicking preeclampsia.[330] The levels of soluble Flt-1 and endoglin were recently shown to be potentiated in HO-1-deficient mice as well as wild-type mouse placental explants treated with an HO-1 inhibitor (SnPPIX).[331] Correspondingly, administration of a CO donor (CORM-2) decreased soluble Flt-1 released from cultured HUVECs, whereas adenoviral overexpression of HO-1 in placental explants reduced soluble endoglin.[331]

The introduction of HO-1 siRNA to mice bearing hepatocellular carcinomas produced a decrease in angiogenesis in the tumors as well as an overall decrease in tumor size growth rate.[332] This suggests that HO-1 gene suppression could be an effective antiangiogenic therapy for cancer.

7.8. What are the Targets of CO in Angiogenesis?

No unique direct target of CO has been found that mediates angiogenesis. This is not to mean that there is none, and further effort is needed on this topic. Like NO, CO signaling in vascular cells has been attributed to activation of the heme protein sGC.[333] However, CO is only a weak activator of sGC and requires levels that are 2 orders of magnitude more than NO to elicit a signal. Consistent with this, the inhibition of sGC has no effect on the anti-inflammatory actions of CO in macrophages.[334] Aside from a direct activation of sGC, CO could increase the concentration of NO in cells by limiting the scavenging of NO by other heme proteins or $O_2^{\bullet -}$. As stated above, NO reacts with $O_2^{\bullet -}$ at nearly diffusion-controlled rates to form OONO−, which is viewed here as a potential sink for NO but is also a controversial physiological oxidant.[335,336] CO can potentially bind reduced heme proteins to limit their ability to scavenge NO. More importantly, it has been shown that heme oxygenase (1 and 2) derived CO up-regulates the expression of ecSOD[337,338] to limit the concentration of superoxide. This may be more significant in terms of the interaction of CO and H_2O_2 angiogenic signaling. This would allow CO to act as a feedback stimulator of H_2O_2 signaling from the NADPH oxidases by providing more of the component necessary to convert their product into an angiogenic signal. In addition, CO could also directly mediate the production of $O_2^{\bullet -}$ (and ultimately H_2O_2) from the mitochondria. Paradoxically, CO was reported to mediate its anti-inflammatory effects by binding cytochrome *c* oxidase (complex IV of the mitochondrial electron-transport chain), increasing the flux of ROS production from the mitochondria.[339] CO competes with oxygen for binding to cytochrome *c* oxidase with a K_i of 0.3 μM, and 20 μM CO inhibited cellular respiration 40% in the presence of 20 μM O_2.[340,341] A role for mitochondrial-derived H_2O_2 in angiogenesis is suggested in work by Chandel et al.[342] Hep3B cells deficient in mitochondria (termed ρ^0) failed to produce VEGF and erythropoietin in response to hypoxia.[342] Interestingly, these cells retained the ability to produce these angiogenic factors upon H_2O_2

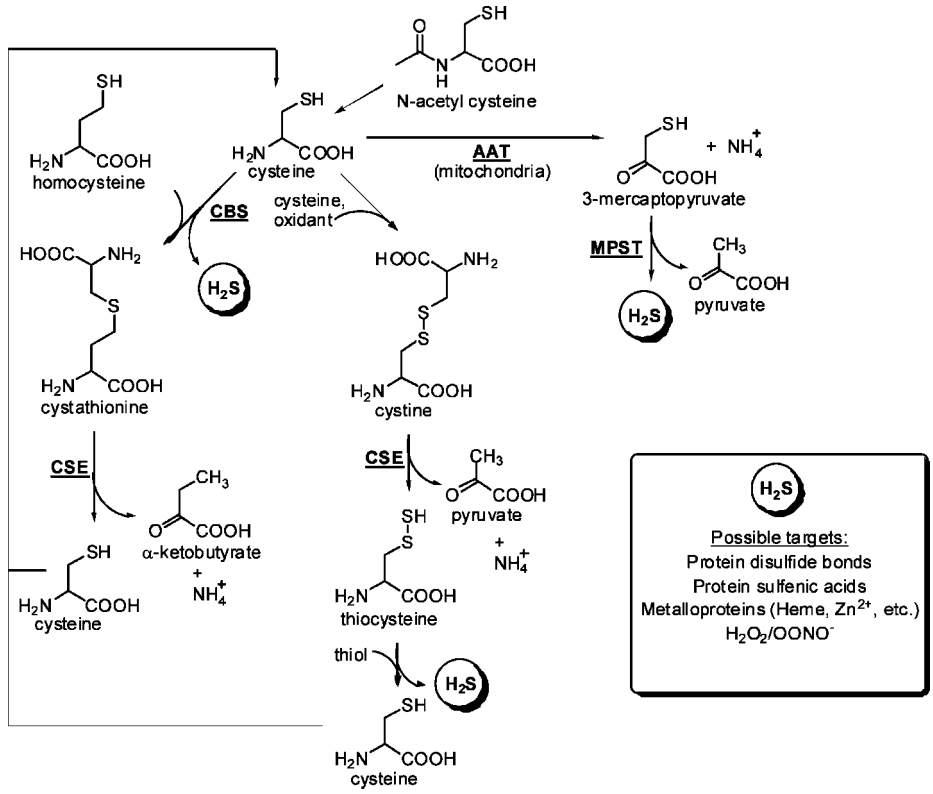

Figure 8. H_2S biosynthesis. H_2S is synthesized by 3 different enzymes: cystathionine beta-synthase (CBS), cystathionine γ-lyase (CSE), and mercaptopyruvate sulfurtransferase (MPST). CBS condenses cysteine and homocysteine to make cystathionine and H_2S. CSE takes cystine and converts it to thiocysteine, pyruvate, and ammonia. Thiocysteine can be reduced by another equivalent of thiol to cysteine and H_2S. Cysteine, converted in the mitochondria by aspartate aminotransferase (AAT) to 3-mercaptopyruvate, is converted to H_2S, pyruvate, and ammonia by MPST.

stimulation with $CoCl_2$, indicating that there are multiple ROS-generating systems with a common target.[342] Connor et al.,[343] using an SOD2 overexpressing fibrosarcoma cell line, showed that increases in mitochondrial-derived H_2O_2 induced VEGF synthesis through inhibition of the redox-active tumor-suppressing phosphatase, PTEN (phosphatase and tensin homologue deleted from chromosome 10). PTEN attenuates PI3K signaling and Akt activation, leading to HIF1-α stabilization and VEGF production.[343] In addition to this, cells producing a higher steady-state level of mitochondrial H_2O_2 produced greater cell sprouting in an in vitro angiogenesis assay as well as an in vivo angiogenic assay using the chicken chorioallantoic membrane.[343]

The fact that CO is involved in angiogenic signaling is now well-established. However, the direct targets of CO-mediated angiogenic signaling and their relative contributions to the overall process remain elusive, although actively researched. The recently defined role of a hydrogen sulfide (H_2S) biosynthetic enzyme as a physiological CO target is discussed in section 8. Further research is also needed to clarify the broader implications of CO crosstalk with NO and ROS.

8. What is the Contribution of Hydrogen Sulfide?

Until recently, H_2S was considered only as a toxic environmental pollutant and associated with the smell of rotten eggs. Indeed, H_2S is more toxic than CO or hydrogen cyanide.[314] Like its so-called "gasotransmitter" cousins, H_2S is now gaining research interest based on newly reported physiological signaling properties (reviewed in refs 344−347. H_2S is categorized with CO and NO because it is a water-soluble gas at room temperature and pressure, though all of the signal transduction properties of the "gasotransmitters" are derived from their solute form. Unlike CO and NO, however, H_2S has an acidic proton with a pK_a of 6.8, making the anionic conjugate base the predominant form (~80%) in biological fluids at physiological pH (7.4). However, equilibrium with the highly lipophilic protonated form enables it to freely penetrate cell membranes.

8.1. H_2S Biosynthesis

H_2S is synthesized endogenously by three enzymes: cystathionine β-synthase (CBS), 3-mercaptopyruvate sulfurtransferase, and cystathionine γ-lyase (CSE)[348] (Figure 8). Two of these enzymes use L-cysteine as a substrate to make H_2S but were discovered in different locations and named for a different reaction. CBS is the primary H_2S producer in the brain, where it normally makes cystathionine by condensing serine and homocysteine.[349] H_2S production from CBS is stimulated 2−3 fold by Ca^{2+}[347] and 2-fold by S-adenosylmethionine[349] and requires pyridoxyl phosphate. CBS also contains a noncatalytic heme iron to which CO and NO have been shown to bind and inhibit.[350] However, CO binds with a 200-fold higher affinity than NO and is much more likely to regulate CBS under physiological conditions.[350] CBS activity can result in physiological H_2S concentrations as high as 50−160 μM in mammalian brains.[351] CSE, on the other hand, is largely found in peripheral tissues such as the kidney, lungs, and vasculature,[352] while the liver contains large amounts of both enzymes.[353] In the rat vasculature, the highest amount of H_2S is produced by the tail artery followed by the aorta and the mesenteric artery.[354]

8.2. H_2S Signaling Targets

Though H_2S is ubiquitously produced and apparently in high concentration in some vascular tissues, there is no established signaling mechanism. Confounding this, many of its biological effects remain controversial. H_2S can stimulate or block apoptosis; can be produced too much or too little in myocardial ischemia; can stimulate or inhibit cell proliferation; and can be pro- or anti-inflammatory in a mouse model of edema. It is more firmly established in the cardiovascular system that H_2S acts as a vasodilator relaxing aorta, portal vein, corpus cavernosum, mesenteric, and hepatic arteries, but not coronary arteries in vitro.[344] H_2S given intravenously to whole animals decreases blood pressure in a dose-dependent manner.[355] The mechanism of this hypotensive activity received much attention and was determined to be due to opening of ATP-sensitive K^+ (K_{ATP}) channels in smooth muscle cells by H_2S.[351] Although the direct effect of H_2S on K_{ATP} channels is not yet known, it is speculated that H_2S may reduce a key disulfide that regulates the channel.[356] A role of H_2S as a physiologic vasorelaxant was recently established using $CSE^{-/-}$ and $CSE^{-/+}$ transgenic mice.[357] The lack of CSE-generated H_2S resulted in hypertensive mice and significantly increased the level of plasma homocysteine. This work clearly establishes H_2S as an endothelium-dependent vasodilator that is sensitive to changes in intracellular calcium, much akin to NO/NOS regulation.

Two reports show direct angiogenic effects of H_2S. One work concluded that H_2S is proangiogenic, having a maximum effect at 10−20 μM.[358] H_2S increased cell growth and migration in cultured endothelial cells in a manner that was dependent on Akt and PI3K. The effect of H_2S was also independent of increases in VEGF, FGF, angiopoietin-1, or NO metabolites. Mice treated with 10 and 50 mM kg^{-1} day^{-1} showed increased angiogenesis in vivo in Matrigel implants.[358] In the second study, H_2S also stimulated endothelial cell proliferation in vitro, but similar concentrations inhibited vascular outgrowth in a muscle explant angiogenesis assay.[359] The latter result suggests that H_2S has effects on angiogenesis that extend beyond its direct effects on endothelium.

Angiotensin II (Ang II) is a potent stimulator of NADPH oxidase in VSMC,[221,242] endothelial cells,[360,361] adventitia,[362] and cardiac myocytes.[363,364] By binding to different receptors, Ang-II can either stimulate or inhibit angiogenesis.[365] Ang II is the product of angiotensin-converting enzyme, a zinc metallopeptidase that cleaves angiotensin I to angiotensin II (a vasoconstrictor) while also degrading bradykinin (a vasodilator). H_2S is an inhibitor ($K_I \approx 100$ μM) of angiotensin-converting enzyme in HUVEC.[366] The effect of H_2S was abrogated in the presence of Zn^{2+}, indicating that H_2S may be interacting with the Zn^{2+}-containing active site of angiotensin-converting enzyme.[366] Consistent with this is the fact that H_2S biosynthesis is increased in tissues of streptozotocin-induced diabetic rats,[367] correlating the impaired vascular responses and angiogenesis/wound healing attributed to diabetes.

8.3. H_2S and Down Syndrome

Down syndrome, which is a result of having an additional copy of chromosome 21, has been linked to overproduction of H_2S due to CBS being encoded on this chromosome.[368,369] Overproduction of H_2S has been hypothesized to be a cause of the gradual mental retardation associated with afflicted individuals.[370] Interestingly, the incidence of solid tumors and hemangiomas is significantly lower in Down syndrome individuals, indicating that they may have impaired angiogenesis.[371] Excess H_2S may not be the only cause because others have attributed impaired angiogenesis to an extra copy of the type XVIII collagen gene, which increases the circulating concentration of endostatin.[372]

8.4. H_2S and Homocysteinemia

Hyperhomocysteinemia, a condition of elevated homocysteine in the blood, may be a marker of impaired CSE activity or expression and, therefore, of reduced H_2S biosynthesis (Figure 8). This is evident in the $CSE^{-/-}$ transgenic mouse where H_2S levels are down 80%, while plasma homocysteine is up over 18-fold.[357] Indeed H_2S and homocysteine are associated with opposing effects in cardiovascular studies.[373] Homocysteine is elevated following balloon injury (a model of angioplastic restenosis) in rats as H_2S and CSE levels are lowered, leading to significant neointimal thickening.[374] Introduction of H_2S relieved the effects of balloon injury induced hyperplasia, supporting its role as an antiangiogenic mediator.[374] Also, lowering plasma homocysteine levels in patients with peripheral artery disease and diabetes mellitus resulted in a significant lowering of circulating VEGF without significantly altering endostatin levels.[375] Many additional reports associate changes in homocysteine levels with angiogenic and vascular outcomes, highlighting the potentially integral yet poorly defined role played by the transsulfuration pathway.

Despite some evidence supporting a role for H_2S in angiogenesis, this remains an infant field. In a broader context, H_2S is gaining traction due to exciting cardiovascular and neurological effects of the "third gasotransmitter". It will be important to define how H_2S integrates into the signaling actions of the other agents (NO, CO, ROS/H_2O_2, etc.) discussed here in terms of angiogenesis but also in more broad physiological terms. For instance, studies have reported the regulation of CO production by H_2S in activated macrophages[376] or the mutual modulation of NO and H_2S in the vasculature.[351,377]

9. HNO and Angiogenesis

There has been recent excitement about the possible physiological generation of the one electron-reduced and protonated form of NO, nitroxyl (HNO) (most recently reviewed in ref 378. HNO has important cardiovascular effects that are both shared[379,380] and distinct from NO.[380−382] It is produced from NOS in vitro as well as by direct reduction of NO. Another often overlooked pathway is the reaction of an *S*-nitrosothiol with another thiol:

$$\mathrm{RSNO} + \mathrm{R'SH} \rightarrow \mathrm{HNO} + \mathrm{RSSR'} \qquad (7)$$

However, because of the lack of direct detection methods, its physiological generation has not been conclusively demonstrated. Despite this, a number of HNO donor molecules have been used to explore its pharmacological functions as well as to establish biological reactivity that departs from NO.[379] HNO prefers to bind to ferric heme proteins but will form complexes with ferrous hemes.[383] HNO is extremely thiophilic, leading to reversible (*N*-hydroxysulfonamide) and irreversible (sulfinamide) modifications.[384] Unlike NO, it does not react with superoxide.[385] Consistently, the main biological targets of HNO are low pK_a thiols[386] and ferric heme proteins,[386] although it can directly bind and

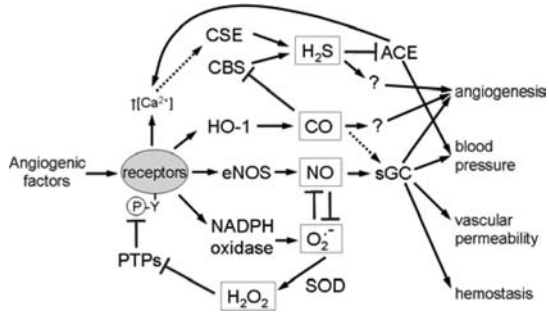

Figure 9. Cross-talk between H_2S, CO, NO, $O_2^{\cdot -}$, and H_2O_2 in vascular angiogenic signaling. Binding of an angiogenic factor to its cell-surface receptor results in receptor tyrosine phosphorylation and downstream kinase activation of HO-1 (producing CO), eNOS (producing NO), and NADPH oxidase (producing $O_2^{\cdot -}$). Receptor activation can also increase intracellular calcium levels, activating CSE (producing H_2S). $O_2^{\cdot -}$ is converted to H_2O_2 by SOD, and this is capable of either direct or indirect inhibition of PTPs that normally attenuate receptor downstream signaling. H_2O_2 provides a reinforcement of the angiogenic signal. $O_2^{\cdot -}$ can also react with and consume NO making $OONO^-$, decreasing the signaling capacity of both NO and $O_2^{\cdot -}$. NO through sGC is a primary regulator of angiogenesis, blood pressure, vascular permeability, and hemostasis. Likewise, CO can bind sGC to regulate the same processes, but with a much lower affinity (pM vs mM). However, CO also regulates angiogenic signaling in a sGC-independent manner. H_2S is an inhibitor of angiotensin converting enzyme (ACE) with associated effects on blood pressure and has an ill-defined role in angiogenesis. Increase in CO by HO-1 induction could also decrease the levels of H_2S derived from CBS.

activate the ferrous heme protein sGC to elicit NO-like activation (T.W.M. unpublished results).

Although HNO differs empirically from NO in only a proton and an electron, it may elicit opposite angiogenic effects from NO. Mice treated with an HNO donor showed decreases in xenograft tumor size as well as decreased tumor vessel density.[387] Accordingly, circulating VEGF and HIF1α levels were decreased. The authors speculate that the effect of HNO on angiogenesis is dependent on potent inhibition of glycolysis (GAPDH) by HNO, leading to decreased HIF1α and decreases in VEGF.

10. Conclusions

Studies of developmental and tumor angiogenesis have uncovered important signaling roles for gasotransmitters and other redox-active small molecules. They play key roles as cytoplasmic and intercellular mediators of both pro- and antiangiogenic signaling. To date, NO is the best understood of these mediators, and its role in developmental versus pathological angiogenesis is fairly well-understood. Efforts to directly limit NO synthesis using NOS inhibitors have shown some ability to limit tumor angiogenesis in animal models, but the pleiotropic role of NO in cardiovascular physiology to control blood pressure and hemostasis would likely prevent any long-term therapeutic use of such inhibitors in cancer patients. However, recognizing that NO signaling plays several roles in the cardiovascular system may be crucial to developing more effective therapeutic angiogenesis inhibitors. All of the currently FDA approved antiangiogenic drugs have hypertensive and prothrombotic side effects,[134,388] which are shared by some endogenous angiogenesis inhibitors,[165,389] and the role of NO signaling in the etiology of these side effects is increasingly being recognized (Figure 9). Defining the angiogenesis-specific versus general targets of cardiovascular NO signaling may allow tumor angiogenesis to be controlled without these adverse side effects. One recent example that applies this strategy employed NO-independent vasodilators to overcome the hypertensive activity of an experimental VEGFR2 kinase inhibitor without impairing its ability to inhibit tumor angiogenesis.[136]

Efforts to define the roles of other members of this family in angiogenesis are at much earlier stages than for NO. Indeed, to fully understand and pharmacologically control pathologic angiogenesis, we may need to both define their individual targets and integrate all of these molecules into a signaling network. This review has mentioned a few such connections, and it may be instructive for future research efforts to highlight what is known about cross-talk between their signaling pathways (Figure 9). At physiological concentrations of NO relevant to stimulating angiogenesis, the primary target of NO is clearly sGC. $O_2^{\cdot -}$ plays a primary role in proangiogenic signaling by providing positive feedback through PTP inactivation to prolong signaling through Tyr-kinase receptors, which in turn prolongs the synthesis of NO via eNOS. Although NO and $O_2^{\cdot -}$ can react to neutralize each other, it is unclear that they achieve sufficient concentrations or colocalization in vascular cells for this to provide significant negative cross-talk. Although it is clear that CO plays an important role in angiogenesis, the mechanism is less clear. Its direct activation of sGC is weak, so other undefined targets may play a dominant role in angiogenic signaling. One potential indirect pathway is via its inhibition of H_2S synthesis via CBS. Physiological CO levels are consistent with this mechanism, yet current data suggests that CSE rather than CBS is the major source of H_2S synthesis in the vasculature. H_2S in turn can clearly inhibit ACE at physiological concentrations, and the resulting loss of Ang II could account for the effects of H_2S on blood pressure. It is unclear whether the same pathway mediates the reported effects of H_2S on angiogenesis or whether another target needs to be identified for this function. Inhibition of ACE would alter Ang II, which is known to control NO synthesis via the AT2 receptor. Thus, angiogenic signaling of H_2S could be mediated by NO.

11. Acknowledgments

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12. Abbreviations

ACE	Angiotensin converting enzyme
Ang	angiopoietin
Ang II	Angiotensin II
CBS	cystathionine β-synthase
CSE	cystathionine γ-lyase
cGK	cGMP-dependent protein kinase
CORM	CO-releasing molecule
CSE	cystathionine γ-lyase
EGFR	epidermal growth factor receptor
EPC	endothelial progenitor cells
FGF2	fibroblast growth factor-2, also known as basic fibroblast growth factor
HO-1	heme oxygenase-1
Hsp90	heat shock protein-90
HUVEC	human umbilical vein endothelial cells
I/R	ischemia/reperfusion
LDL	low-density lipoprotein

MLC2	myosin light chain 2
MMP	matrix metalloproteinase
NOS	nitric oxide synthase
NOX	NADPH oxidase
PDE	phosphodiesterase
PI3K	phosphatidylinositol 3-kinase
PlGF	placental growth factor
PPARγ	peroxisome proliferator-activated receptor-γ
PPIX	protoporphyrin-IX
Prx	peroxiredoxin
PTP	protein tyrosine phosphatase
ROS	reactive oxygen species
S1P	sphingosine 1-phosphate
SDF-1	stromal cell-derived growth factor 1
sGC	soluble guanylate cyclase
TNFα	tumor necrosis factor α
TSP	thrombospondin
VEGF	vascular endothelial growth factor
VSMC	vascular smooth muscle cells

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