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Reactive Oxygen Species Drives Myocardial Angiogenesis?

NILANJANA MAULIK

ABSTRACT

Neovascularization, the natural physiological process of formation of new blood vessels, is extremely important for ameliorating the function of the heart that undergoes ischemic stress. This process is potentially important for the treatment of ischemic heart and limb diseases, which includes formation of capillaries (angiogenesis) and collateral arteries. Ischemia or coronary artery occlusion induces vascular endothelial growth factor (VEGF) in the experimental rat myocardial infarction model, and this molecule encourages development of coronary collateral circulation and retention of the blood supply to the ischemic area. Restoration of the blood supply to the ischemic area prevents cardiomyocyte death and cardiac remodeling. Among the various triggers and enhancers of angiogenesis, hypoxic or ischemic preconditioning, as well as pharmacologic agents such as statin and resveratrol, have been identified as important stimuli for the induction of new vessel growth. It has already been demonstrated that the VEGF family and its receptor system is the fundamental regulator in the redox cell signaling of angiogenesis. This review article will focus on the role of reactive oxygen species in the process of myocardial angiogenesis. *Antioxid. Redox Signal.* 8, 2161–2168.

INTRODUCTION

The central role of vascular endothelial growth factor (VEGF) in angiogenesis in health and disease makes it attractive as a therapeutic target for anti-angiogenic drugs and as a pro-angiogenic cytokine for the treatment of ischemic heart disease. Whereas VEGF binds to two receptor protein tyrosine kinases, VEGFR1 (Flt-1) and VEGFR2 (Flk-1/KDR), most biological functions of VEGF are mediated by Flk-1. However, studies from our laboratory have already confirmed the significance of Flt-1 receptors also in ischemic preconditioned-mediated cardioprotection (17) involving downregulation of several important genes in Flt-1-knockout mice (Flt-1$^{-/-}$) in an ischemic reperfused model (Table 1). The exact mechanism of VEGF signaling via Flt-1 is yet to be determined and requires well-designed studies. Recently, we demonstrated for the first time the ability of ischemic preconditioning (IPC) to induce angiogenesis in infarcted myocardium along with the activation of several transcription factors such as stat3, Pax-5, NF-κB, TFIID, SP1, and reduction of VEGF-mediated vascular permeability by inhibition of c-src in ischemic preconditioned myocardium, thereby reducing ischemic injury in the rat myocardial infarction (MI) model (17). However, the mechanism by which activation of VEGFRs elicits these cellular events is not fully understood.

Reactive oxygen species (ROS) stimulate angiogenic responses in ischemic reperfused hearts. Several sources of ROS have been identified in the cell, the most significant one being the mitochondrial respiratory chain. In the cytosol, other intracellular sources of ROS include xanthine oxidase and cytochrome P450, as well as membrane NADPH oxidase and nitric oxide synthase (40, 60). We have already documented that *in vitro* short exposure to hypoxia/reoxygenation, either directly or indirectly, produces ROS in human coronary arteriolar endothelial cells that induce oxidative stress, which is associated with increased VEGF and angiogenesis or tuborogenesis (71). It is well documented that ROS can both cause tissue injury and promote tissue repair by promoting angiogenesis. Reactive oxygen species derived from gp91^{phox} (NOX2)-containing NAD(P)H oxidase are involved in angiogenesis in mouse sponge models, as well as in VEGF signaling in

Molecular Cardiology and Angiogenesis Laboratory, Department of Surgery, University of Connecticut Medical Center, Farmington, Connecticut.

Table 1. Microarray Analysis Demonstrating Significant Number of Downregulated Myocardial Genes in Flt-1$^{-/+}$ Mice

Gene name	*Ratio (Flt-1$^{+/-}$/WT)*
GRO1 oncogene (Gro1)	0.13
Exportin 4	0.16
V kappa 21–11	0.23
Anxa7	0.24
IKKβ	0.27
Human zinc finger protein (ZNF139)	0.28
CSF-1	0.29
Ubiquitin-conjugating enzyme	0.32
MgcRacGAP mRNA for GTPase activating protein	0.38
Platelet derived growth factor, alpha (PDGFα)	0.41
Trinucleotide repeat containing 7 (TNRC7)	0.42
Asparagine synthetase mRNA	0.43
H3051H08	0.44
H3112H12	0.44
Zinc finger protein 57 (ZFP57)	0.47
Interferon gamma induced GTPase	0.49
Heat shock transcription factor 2 binding protein (HSF2BP)	0.52
Inducible 6-phosphofructo-2-kinase mRNA	0.52
HSP84 kDa	0.57
HSP70 kDa	0.60
JAK2	0.63
Beta-2 microglobulin (B2m)	0.63

cultured endothelial cells (16). The role of gp91^{phox}-derived ROS in neovascularization in response to tissue ischemia is not well studied, although impairment of neovascularization recently has been demonstrated in gp91phox $^{-/-}$ mice with hindlimb ischemia (62). There are also no reports describing which pathways are predominant, as study of the role of ROS in angiogenesis is relatively new. We think the important pathway will depend on the relative contribution of Flk-1-dependent pathways activated after ischemic/hypoxic stress followed by reperfusion. In addition, there may be additional pathways that are Flk-1 dependent but ROS independent.

ROLE OF REACTIVE OXYGEN SPECIES IN ANGIOGENESIS

Oxygen homeostasis is of critical importance for maintaining the viability of all tissues. Lack of sufficient tissue oxygenation is predominantly caused by impaired blood flow. Attempts to restore normal oxygen levels after episodes of hypoxia or ischemia result in the generation of various types of free radicals, such as superoxide anions, hydrogen peroxide, and hydroxyl radical, collectively known as reactive oxygen species (ROS). Although lack of adequate oxygen (*e.g.*, hypoxia) is an initiator of various diseases, it also can trigger a unique "repair" mechanism, that acts as an important inducer of angiogenesis. ROS play a very important role in signaling pathways stimulated by growth factors in vascular cells. Recent reports suggest that ROS, such as superoxide anions (O_2-), play an important role in mediating signals initiated by growth factors and inflammatory cytokines (11). In this regard, we have previously shown that hypoxic preconditioning (hypoxia/reoxygenation) mediates the activation of NF-κB in rat myocardium and human coronary arteriolar endothelial cells (38, 39, 50, 54, 71)

Reactive oxygen species such as superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) are involved in the signaling pathways mediating many stress and growth responses, including angiogenesis (31). In endothelial cells, H_2O_2 stimulates cell migration and proliferation (43). Hypoxia/reoxygenation, which produces ROS, elicits capillary tube formation in human coronary arteriolar cells (14). *In vivo*, elevated oxidative stress directly correlates with neovascularization and VEGF expression in the retina of diabetics (49) and in aortic plaque of models of atherosclerosis (64). Hypoxia is a strong inducer for VEGF expression both *in vivo* and *in vitro* (20). Fibroblast growth factor (FGF) mRNA is increased also in the brains of animals exposed to hypoxia (10). H_2O_2-mediated strong VEGF gene expression was also demonstrated in the rat-derived endothelial cells (19). Several metabolic pathways in mammalian cells, such as xanthine/xanthine oxidase, NADPH oxidase, mitochondrial and microsomal electron transport chains, and cyclooxygenase pathway can generate ROS and H_2O_2. ROS and H_2O_2 at high concentrations are highly toxic to cells. However, at low concentrations, ROS play various physiological roles such as regulating gene expression and cell proliferation. They are also pivotal as an intracellular second messenger. H_2O_2 was found to induce VEGF mRNA in a dose-dependent protein kinase c (PKC)-dependent manner (56). Moreover, Shih *et al.* (56) suggested that PKC upregulates VEGF mRNA in human glioblastoma cells through the stabilization of VEGF mRNA. The temporal change of VEGF mRNA (28) supports the hypothesis because ischemic preconditioning (IPC) rapidly and greatly upregulated VEGF mRNA at 3–12 h after infarction, whereas there was a smaller extent of VEGF mRNA induction irrespective of the IPC procedure at 1–3 days after infarction. This report also showed that IPC might enhance VEGF gene expression and angiogenesis through nuclear translocation of PKCϵ in the infarcted myocardium.

VEGF was rapidly induced by transient ischemia in the heart. A single episode of ischemia by abrupt ligation of a coronary artery led to a prolonged activation of VEGF mRNA in the rat heart. After 1 h of ligation, expression of VEGF mRNA, as determined by quantitative Northern analysis of the entire heart, increased by 2.5-fold. Expression of Flk-1 (VEGFR-2) followed a similar pattern, reaching a peak after a 3.7-fold increase at 1 h and then reduced slowly. Flt-1 (VEGFR-1) also increased following left anterior descending (LAD) coronary artery ligation (25). Since increased production of ROS has been well documented in myocardial ischemia and reperfusion, it is possible that such upregulation of VEGF is mediated by ROS. Several studies demonstrated the presence of AP-1 and NF-κB binding sites in the promoters of human and mouse VEGF genes. In stimulated endothelial cells, the oncogenic transcription factors c-ets-1 and c-rel/NF-κB function to induce specific gene expression (58). In these activated endothelial cells, c-ets-1

regulates the expression of a number of genes involved in matrix degradation, including collagenase, stromelysin, and urokinase-type plasminogen activator.

Reactive oxygen species derived from NAD(P)H oxidase are critically important in many aspects of vascular cell regulation, and both the small GTPase Rac 1 and gp91phox are critical components of the endothelial NAD(P)H oxidase complex. A major source of endothelial $O_2^{-\bullet}$ generation is NAD(P)H oxidase, which consists of a plasma membrane spanning flavocytochrome b558 composed of gp91^{phox} and p22^{phox}, and cytosolic components p47^{phox} and p67^{phox}. The small molecular weight G protein Rac is also necessary for assembly of the active NAD(P)H oxidase complex (46). Recently it was demonstrated that a gp91^{phox} containing NAD(P)H oxidase was a major source of ROS in vascular endothelial cells (4), and that Rac 1 are critical components of endothelial NAD(P)H oxidase. *In vitro* studies suggest that ROS derived from gp91^{phox} containing NAD(P)H oxidase are important in VEGF signaling and angiogenesis. More elaborate study is necessary to document the components of NAD(P)H oxidase as potential targets for angiogenic therapy in ischemic heart disease. VEGF-induced cell signaling and angiogenesis are tightly controlled by the reduction/oxidation environment at the level of VEGF receptors and will provide novel insights into NAD(P)H oxidase as a potential therapeutic target for myocardial angiogenesis. Our results in the future may suggest that ROS derived from gp91^{phox} containing NAD(P)H oxidase are important in VEGF signaling after ischemia-reperfusion to initiate the repair mechanism followed by angiogenesis.

VEGF SYSTEM DURING ISCHEMIC STRESS

The process of angiogenesis is regulated by signals obtained from the transmembrane receptor tyrosine kinases (RTKs) and nonreceptor tyrosine kinases of endothelial cells. Flk-1/KDR and Flt-1 are two such RTKs, which, together with their ligand VEGF, have been shown to control blood vessel development during embryogenesis (55, 59). This receptor/ligand system augments neovascularization (3, 5, 23, 32, 43, 51). Studies in rat myocardial infarction models also demonstrated significant induction of VEGF (275%), Flk-1 (375%), and Flt-1 (400%) mRNA expression throughout the entire heart after 1 h of infarction (34). A great deal of attention has been directed toward studies of VEGF expression and its function in myocardial ischemia/hypoxia (6, 21, 22, 27, 47, 48) and relatively little is known regarding the mechanism of its receptors, Flk-1 and Flt-1. VEGF is the only known ligand for Flk-1, whereas Flt-1 is able to bind to placental growth factor in addition to VEGF.

Embryological studies have demonstrated abundance of Flk-1 in human lung tissues, while Flt-1 was found to be abundant in the heart, lung, and kidneys (27). Careful investigations demonstrated functional differences between Flk-1 and Flt-1 in endothelial cells. In the developing human heart, both receptors were expressed in myocardial capillaries, and stimulated intracellular calcium flux along with VEGF stimulation. Genetically manipulated Flk-1 knockout (homozygous) studies demonstrated early embryonic death due to inhibition of vasculogenesis, whereas in another study, homozygous Flt-1 disruption caused failure to assemble normal formation of vascular channels (55). In another important observation it was found that Flt-1 was expressed in endothelium of both large and small vessels, whereas Flk-1 expression was restricted to only small vessels (34). One of our recent studies demonstrated a myocardial distribution pattern of Flk-1 and Flt-1 after rats were exposed to whole body hypoxia followed by 24 h of reoxygenation. Intense staining was observed along the capillaries, in addition to strong localization around the coronary arteries (55). We have also documented the intensity of staining for both the receptors, which increased significantly in the hypoxia/reoxygenation group compared to the corresponding normoxic control group (55). Western blot analysis also documented similar results. In other words, in a systemic whole body hypoxic rat model, we found significant abundance of Flk-1 protein expression in the 1 h hypoxia group, that remained elevated in the 2 h group (hypoxic), but was reduced in the 4 h hypoxic group, followed by 24 h reoxygenation, suggesting a role in the initial rather than the later stages of the early angiogenic process. In contrast, induction of Flt-1 protein expression was increased in the 1 h hypoxia group and continued to be elevated even in the 4 h hypoxia group, indicating a more continuous role in the early angiogenic process (55). We have also documented significant improvements in myocardial function along with increased capillary and arteriolar density following induction of survival factors VEGF, Bcl-2, and survivin in the setting of the fully established chronic rat myocardial infarction model subjected to ischemic preconditioning (17, 37). Mammalian hearts can be adapted to ischemia by repeatedly subjecting it to short-term reversible ischemia, each followed by short duration of reperfusion. This phenomenon is known as "ischemic preconditioning (IPC)" (33). Substantial evidence exists to support the notion that oxygen-derived free radicals are generated during reperfusion of ischemic myocardium, resulting in development of oxidative stress.

Ischemia induces the angiopoietin/Tie receptor system in a focal cerebral ischemia model (7). Myocardial adaptation to ischemic stress in stunned pig myocardium demonstrated the induction of c-jun, c-fos, Egr-1, and jun-B that may be involved in the repair process of angiogenesis (15). Adenosine is known to limit the degree of vascular injury during ischemia and reperfusion by inhibition of oxygen free radical release that prevents endothelial cell damage. This may help preserve endothelial cell function and microvascular perfusion (29). Recently we demonstrated that *in vivo* brief repetitive cycles of coronary artery occlusion (5 min), followed by short duration of reperfusion (10 min), triggered myocardial angiogenesis at the capillary and arteriolar levels, which corroborated the improved myocardial contractile function (17). We concluded that ischemic preconditioning-mediated VEGF expression is cardioprotective and VEGF triggers its cardioprotective signal via its receptors Flt-1 and Flk-1 (Fig. 1). However, to date, there has been relatively little information regarding physiological control of angiogenesis by these two VEGF receptors during myocardial protection. Two other angiogenic factors, the angiopoietins 1 and 2 (Ang-1 and Ang-2),

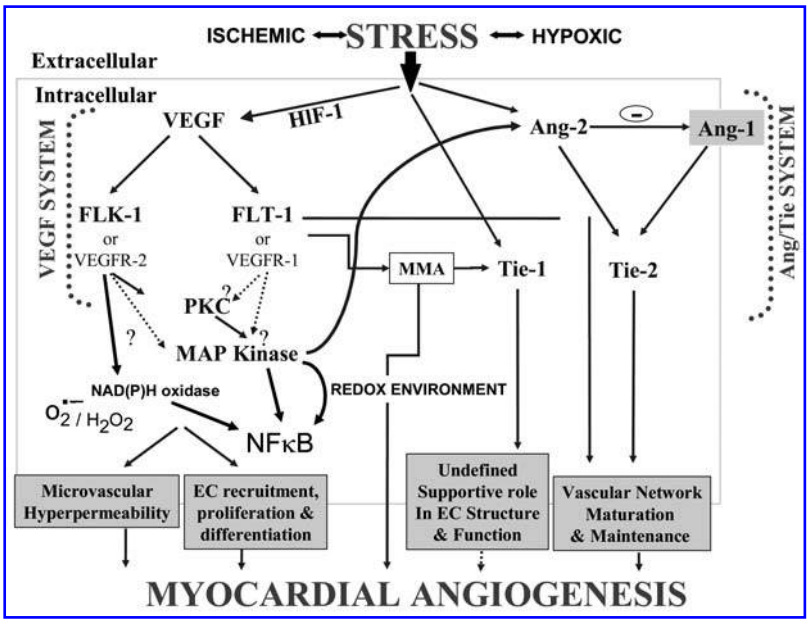

FIG. 1. Schematic diagram showing VEGF and angiopoietin-Tie system involved in cardioprotection. The stimulation of myocardial angiogenesis by stress such as ischemic/hypoxia may constitute a potential basis for a possible more long-lived adaptive response to stress afforded by preconditioning stimuli by upregulating downstream signaling molecule (such as NADPH, PKC, MAP kinases, and NF-κB).

regulate the maturation of new blood vessels from proliferated endothelial cells (69). Tie-1 and Tie-2 comprise another family of endothelial specific receptor tyrosine kinases, Ang-1 and Ang-2 being the specific ligands for Tie-2.

VEGF-mediated angiogenesis signaling is widely accepted; however, relatively little is known regarding VEGF-mediated downstream signaling through Flt-1 and/or Flk-1. Evidence suggests that these two tyrosine kinase receptors of VEGF likely serve different functions in endothelial cells. These two receptors were responsible for stimulating intracellular calcium flux and were also found to be phosphorylated in response to VEGF (61). It is likely that both receptors play a significant role in VEGF-mediated angiogenesis in several pathological as well as physiological situations. Thus, we were very interested in documenting the candidate genes involved in VEGF-mediated signaling through its receptors Flk-1 and Flt-1 in ischemic preconditioned-mediated angiogenesis in an MI model. As we know from literature and also from our own work, VEGF, Flk-1, and Flt-1 expression is linked with angiogenesis and expression of the growth factors, and the same receptors cannot initiate angiogenesis without additional factors. In our study, PC-regulated VEGF-mediated cardioprotection in the WT mice determined several signaling molecules that are involved in VEGF-mediated signaling through Flt-1 receptors. There was no inhibition of VEGF expression, as shown in the Flt-$1^{+/-}$ knockout mice, and the extent of the expression of VEGF was found to be the same in both the wild type and Flt-$1^{+/-}$ knockout with the PC group (1). We have demonstrated clearly that NF-κB plays a significant role in angiogenesis and that inhibition of this redox molecule inhibits the capillary and arteriolar density and tuborogenesis *in vivo and in vitro* (51, 71). Real time PCR for CSF-1 also demonstrated significant involvement of PC-mediated cardioprotection in wild-type control compared to Flt-$1^{+/-}$ knockout. CSF-1 binding to its receptor CSF-1R on cells results in tyrosine phosphorylation of the receptor and many other proteins. It plays a significant role in cell survival and cell proliferation. In addition, significant downregulation of CSF was reported in the development of heart failure after MI (70). Therefore, our result demonstrates that VEGF-mediated signaling through Flt-$1^{+/-}$ involves several factors such as NF-κB, CSF-1, HSP 70, and HSP 84. Preconditioning mediated cardioprotection was found to be significantly affected in Flt-$1^{+/-}$ knockout mice as observed by the functional recovery. The functional recovery was not the same in Flt-$1^{+/-}$ as found in the wild type when subjected to PC (1). The infarct size was found to be significantly higher in the preconditioned Flt-$1^{+/-}$ PC myocardium when compared to the wild-type PC control. The microarray as well as real time PCR data demonstrated significant downregulation of JAK2 mRNA in Flt-$1^{+/-}$ knockout myocardium after ischemia and reperfusion (1). Therefore, in our study we found disruption of cardioprotection in Flt-$1^{+/-}$ KO mice when compared to the wild type.

NITRIC OXIDE AND VEGF EXPRESSION

NO significantly contributes to the prosurvival/proangiogenic process of capillary formation and maturation by triggering and transducing cell growth and differentiation via endothelial-constitutive NO synthase (ec-NOS) activation, cyclic GMP (cGMP) elevation, mitogen activated kinase (MAPK) activation, and fibroblast growth factor-2 (FGF-2) expression (13). Nitric oxide constantly accompanies O_2 and/or ROS in most of their physiological activities. NO is a unique messenger in that it is produced in one cell and diffuses into adjacent target cells to activate cytosolic guanylate cyclase-bound heme to generate the NO-heme adduct of guanylate cyclase. NO can readily react with other cellular hemoproteins such as hemoglobin and myoglobin to produce corresponding NO-heme adducts that can rapidly activate

guanylate cyclase (9, 35, 36). Several *in vitro* studies directly established the role of NO in angiogenesis (72, 73). Treatment of HepG2 cells with NO donor SNAP was found to increase VEGF mRNA expression. Guanylate cyclase is likely to be important for NO-mediated VEGF activation (8). There is also considerable evidence that NO downregulates the expression of the VEGF gene (57, 63). Despite several negative observations, activation of angiogenesis in mammalian (human) monocytes is believed to be NO-dependent (30). Indeed, several studies have documented that NO-generating compounds stimulate angiogenesis in human glastoma and hepatoma cells (8). A positive correlation was found between nitric oxide synthase, cGMP levels, and tumor angiogenesis in head and neck cancer (18). However, the role of NO in angiogenesis is still controversial. For example, NO donors were found to inhibit angiogenic activity in the chick chorioallantoic membrane (45) and the growth and metastatic properties of Lewis lung tumor in mice (44). In addition, NO donors inhibit VEGF expression in the arterial wall in response to balloon angioplasty (63), and in rat lungs during acute and chronic hypoxia (5). Transfer of eNOS (66) and iNOS (65) genes resulted in the inhibition of restenosis after balloon angioplasty. In contrast, a recent study showed L-arginine supplementation after balloon angioplasty of rabbit iliac arteries was beneficial for the healing of endothelium, demonstrating the positive role of NO in angiogenesis (52). Human colon cancer cell lines transfected with a NOS gene grow faster and become more vascularized than normal cell lines *in vivo* (24). NO also enhances the proliferation and migration of endothelial cells *in vitro* (72). Thus, a significant number of studies have demonstrated that NO may stimulate the proliferation of endothelial cells while others failed to prove that such a mechanism exists and suggested an inhibitory effect of NO on endothelial proliferation.

THIOREDOXIN–GLUTAREDOXIN REDOX SIGNALING IN VEGF EXPRESSION

Thioredoxin (Trx) appears to play a crucial role in the redox regulation of the ROS signaling during and/or following ischemia/reperfusion. This protein, Trx, is an important component of the cellular defense against cardiac injury. Oxidized thioredoxin was found to be released into the plasma of patients undergoing cardiopulmonary bypass surgery (42). It is one of the major cellular protein disulfide reductases ubiquitously present in mammalian tissues, including myocardium. They possess dithiol/disulfide active sites and can serve as electron donors for enzymes, including thioredoxin peroxidases and ribonucleotide reductases (2). Thioredoxins are critical for redox regulation of protein function and signaling via thiol-redox control. Thioredoxins are reduced by electrons from NADPH via thioredoxin reductase. Recent studies from our laboratory have provided evidence for redox regulation of myocardial ischemia/reperfusion (34). There are two major thioredoxins: Trx1, a cytosolic and nuclear form, and thioredoxin 2 (Trx2), a mitochondrial form. Trx1 is the major thioredoxin-redox protein, which is responsible for most of the biological signals (22, 41). It plays a crucial role in thiol-redox control of cell function through transcription regulation of target genes including that of NF-κB, which controls numerous gene expressions. Today the glutaredoxin and thioredoxin systems are considered to be parallel redox systems. In fact, the absence of cross-reactivity between the redoxins and the respective NADPH-dependent reductase may have special importance in regulation because the systems can operate independently. In mammalian cells, there seems to be cross talk between the thioredoxin system and the glutaredoxins (22). Very recently, we documented resveratrol-mediated induction of Trx-1, which shows sequential activation and expression of HO-1 as well as pro-angiogenic factor and cardioprotective molecule VEGF in both *in vitro* and *in vivo* models. We have shown that adjunctive treatment with SnPP significantly inhibits all the VEGF-induced angiogenic activities of resveratrol and Trx-1 *in vitro* and *in vivo* (Fig. 2) (26). Our study is in agreement with an earlier report showing that overexpression of HO-1 augments the angiogenic effect in endothelial cells (12) and the activation and overexpression of HO-1 leading to the upregulation of VEGF synthesis. We have explored resveratrol-mediated expression of Trx-1, showing cardioprotective effects in the myocardial infarction model with increased perfused capillary density in peri-infarct myocardium, along with improved cardiac function. The cardioprotective effect is significantly attenuated by SnPP (26). It was reported previously that the redox protein Trx-1 increases hypoxia inducible factor-1α (HIF-1α) protein expression under both normoxic and hypoxic conditions. This is found to be associated with augmented VEGF formation and increased tumor angiogenesis *in vivo* (67). The hypoxia inducible factor-1 complex influences the expression of many genes including VEGF (53). Thus, VEGF is implicated as a major angiogenic factor leading to the development of new vessels from pre-existing capillaries. Transfection of cells with human Trx-1 increases the overall production of VEGF in MCF-7

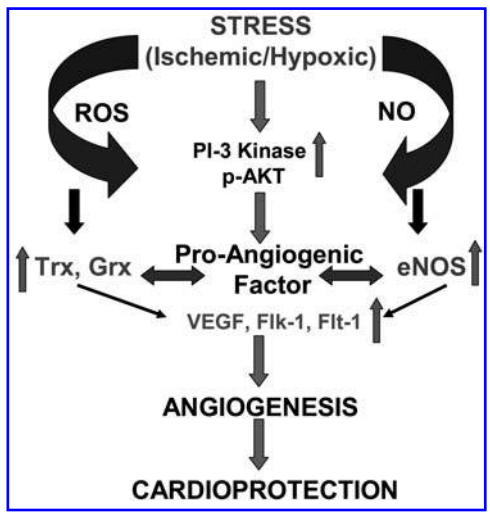

FIG. 2. Schematic diagram suggesting the involvement of reactive oxygen species and redox regulated protein Trx and Grx in angiogenesis, followed by myocardial protection.

breast cancer, HT-29 colon cancer, and WEHI7.2 lymphoma cells [68].

In summary, the angiogenic response in vascular tissue is triggered by ROS signaling in a highly coordinated manner. It appears that massive amounts of ROS produced during ischemia and reperfusion in the vascular tissue, especially in the heart, cause significant injury to cardiomyocytes and endothelial cells. However, during the reperfusion, the same ROS potentiate the repair process and trigger a signal transduction cascade leading to angiogenesis. Significant efforts in this area of research have led to the discovery of a growing number of pro- and anti-angiogenic molecules, some of which are already in clinical trials. However, there are several outstanding questions that must be addressed for successful translation of discoveries from the bench to the bedside. With advances in molecular genetics and the availability of molecular probes, imaging technologies, and therapeutic opportunities, we are now beginning to answer these questions.

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ABBREVIATIONS

APC, adenomatous polyposis coli protein; H_2O_2, hydrogen peroxide; IPC, ischemic preconditioning; MI, myocardial infarction; ROS, reactive oxygen species; $O_2^{-\bullet}$, superoxide anions; VEGF, vascular endothelial cells.

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Address reprint requests to:
Nilanjana Maulik
Molecular Cardiology and Angiogenesis Laboratory
Department of Surgery
University of Connecticut School of Medicine
Farmington, CT 06030–1110

E-mail: nmaulik@neuron.uchc.edu

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