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Potentiation of angiogenic response by ischemic and hypoxic preconditioning of the heart

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Abstract

This review is intended to discuss the newly discovered role of preconditioning which should make it an attractive therapeutic stimulus for repairing the injured myocardium. We recently found that apart from rendering the myocardium tolerant to ischemic reperfusion injury, preconditioning also potentiates angiogenesis. Our study demonstrated for the first time that both ischemic and hypoxic preconditioning triggered myocardial angiogenesis at the capillary and arteriolar levels which nicely corroborated with the improved myocardial contractile function. Hypoxic preconditioning resulted in the stimulation of VEGF, the most potent angiogenic factor known to date. In concert, endothelial cell specific tyrosine kinase receptors, Tie 1, Tie 2 and Flt-1 and Flk-1 were also significantly enhanced in the preconditioned myocardium. The redox-regulated transcription factor NFkB was found to play an essential role in the preconditioning regulation of angiogenesis.

Keywords: ischemic preconditioning - hypoxic preconditioning - angiogenesis - redox signaling - VEGF - NFκB

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Introduction

Coronary heart disease is the primary cause of cardiovascular death. After myocardial infarction (MI), there is a progressive myocardial remodeling characterized by left ventricular (LV) dilation, contractile dysfunction, myocyte hypertrophy and increased matrix protein formation. Ischemic preconditioning provides the most powerful form of endogenous protection against lethal ischemic injury. The classical preconditioning can be induced by a variety of stimuli other than ischemia. Hypoxia [1-3], calcium [4], adenosine agonists [5] α_1-adrenergic agents [6], muscarinic agonists [7], and stretch [8] have been used as preconditioning stimulus to induce tolerance of heart to the subsequent ischemic episode. Hypoxia , an element of ischemia, plays an important role in the cardiovascular system. First described by Neely and Grotyohann in 1984 [9], hypoxic preconditioning, like ischemic preconditioning, can attenuate stunning caused by repeated coronary artery occlusions [1], and enhance postischemic recovery of myocardial function [2]. We have recently shown that hypoxic preconditioning can exert potent cardioprotective effect by upregulating antioxidant reserve of the heart [10]. During the last several years, our laboratory has studied extensively the molecular mechanisms of preconditioning-mediated signal transduction. Our laboratory was the first to demonstrate a preconditioning-mediated signal transduction cascade triggered by tyrosine-kinase and coupled to phospholipase D leading to the activation of MAP kinases [11]. We were also among the first to demonstrate the involvement of p38 MAP kinase in preconditioning resulting in the activation of MAPKAP kinase 2 [12]. Additionally, we documented that translocation and activation of the nuclear transcription factor NFκB plays an essential role in preconditioning [13].

Angiogenic therapy for the human heart is currently being vigorously pursued. In the past ten years, alternative revascularization/angiogenesis strategies have progressed from bench to bed side, focussing on the capillary sprouting and/or growth of new vessels to replace the old. However most of the strategies involves the delivery of growth factors. Very little success with these strategies has been demonstrated so far for various reasons. Very recently, we have demonstrated that both hypoxic as well as ischemic preconditioning, can stimulate myocardial angiogenesis, to an extent sufficient to exert significant cardioprotection in a rat model of myocardial infarction progressing to heart failure as evidenced by increased capillary/arteriolar density and enhanced ventricular contractile functional reserve. The intention of this report is to review how preconditioning potentiates angiogenic response and to clarify the role of redox signaling in the regulation of preconditioning-mediated angiogenesis.

Angiogenesis by ischemic preconditioning

Tissue such as myocardium can be adapted to ischemic stress by repeatedly subjecting it to short-term reversible ischemia each followed by another short duration of reperfusion [12, 13]. This phenomenon, known as ischemic preconditioning or ischemic adaptation, causes the production of oxidative stress leading to the induction of gene expression which is subsequently translated into the development of beneficial proteins responsible for the heart's defence [14, 15].

Substantial evidence exists to support the notion that oxygen-derived free radicals are generated during the reperfusion of ischemic myocardium resulting in the development of oxidative stress. Ischemia was found to induce angiopoietin /Tie receptor system in a focal cerebral ischemia model [16]. 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 repair process of angiogenesis [17]. Adenosine is known to limit the degree of vascular injury during ischemia and reperfusion by inhibition of oxygen free radical release which prevents endothelial cell damage and that might help to preserve endothelial cell function and microvascular perfusion [18]. In our ischemic preconditioned rat myocardial infarction model, we were able to induce angiogenesis after preconditioning. Recently our study 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 (Fig. 1

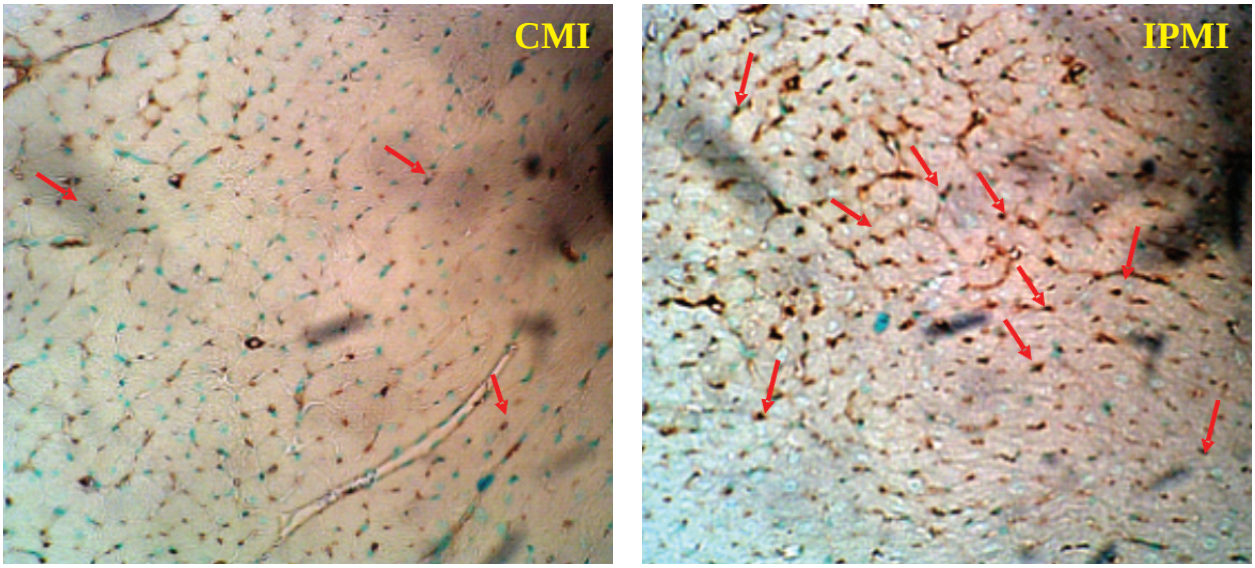

Fig 1 Immunohistochemical analysis of CD 31 labelling of endothelial cells. CMI = Control, MI group; IPMI= Ischemic preconditioned, MI group.

and 2) levels which nicely corroborated with the improved myocardial contractile function [19].

Angiogenesis by hypoxic preconditioning

There are numerous reports on the effects of environmental hypoxic exposure on cardiac pathophysiology. Hypoxia is characterized by inadequate oxygen delivery to the tissue such as myocardium with a resulting imbalance between oxygen demand and energy supply. The possibility that such hyoxic exposure can potentially act as a preconditioning stimulus, however, has not been adequately considered. A strong resemblance exists

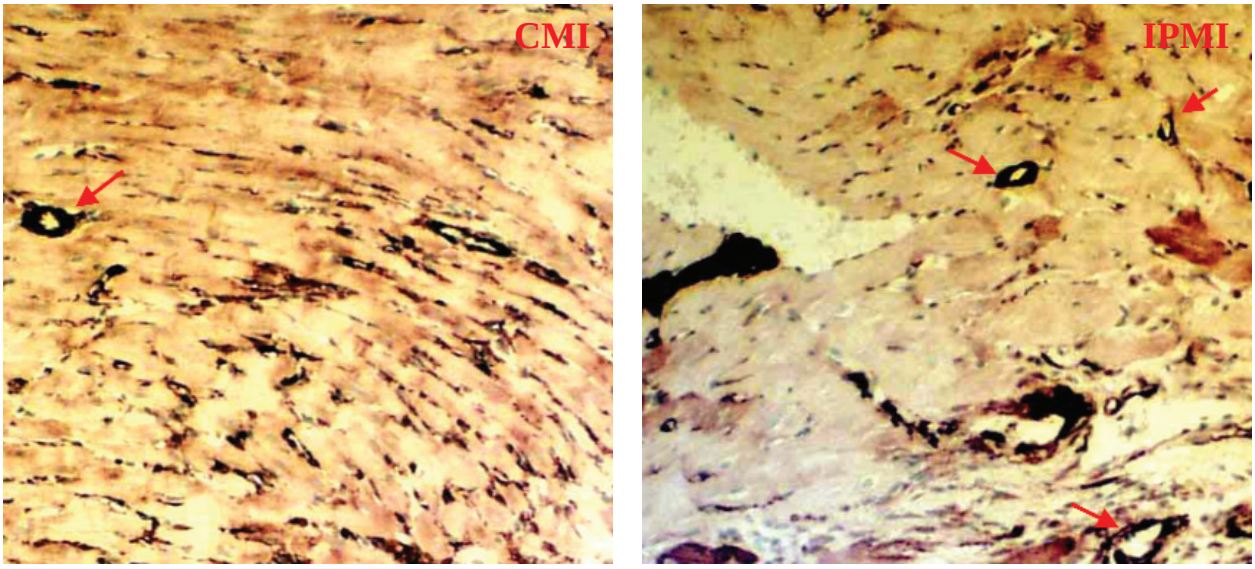

Fig. 2 Immunohistochemical analysis (after one week) of smooth muscle actin labelling of vascular smooth muscle cells. CMI: Control MI group; IPMI= Ischemic preconditioned, MI group.

between the patterns of acute stress response induced by hypoxia/ reoxygenation, ischemia/ reperfusion or any means of generating ROS.

Hypoxia has been found to be the strongest inducer both in vitro and in vivo, of vascular endothelial growth factor (VEGF) which serves as a major angiogen in normal cardiac development [20,21]. Tissue hypoxia exerts a proangiogenic action through various angiogenic factors, the most notable being vascular endothelial growth factor (VEGF). VEGF is mainly associated with initiating the process of angiogenesis through the recruitment and proliferation of endothelial cells.VEGF, a protein coded by a 7-exon gene localized on chromosome 6, serves as a major angiogen in normal cardiac development [22]. We found that non-lethal moderate hypoxic challenge is capable of increasing protein levels of important angiogenic factors and their receptors in the adult rat myocardium. Immunohistochemical analysis of VEGF revealed a diffuse pattern of distribution throughout the ventricular myocardium with strong localization around the coronary arterial wall where both coronary endothelium as well as vascular smooth muscle appeared to stain positive for VEGF (Fig. 3). Hearts obtained from rats which had been subjected to hypoxia followed by a 24 hour period of reoxygenation displayed a progressive increase in intensity of staining for VEGF with increasing durations of hypoxia. Although higher in intensity as compared to control, the distribution pattern remained diffuse and there were no observable areas of localization around capillaries. However, VEGF remained strongly localized around the coronary arteries. VEGF migrated in SDS-PAGE as a dimer of approximately 40 KDa and a monomer of 20 kDa only (Fig. 4). The expression patterns of the two VEGF forms seem similar although the level of the expression pattern seems significantly higher in the case of VEGF dimer. In three different experiments performed in triplicate, significantly increased VEGF expression (about 50% compared to the control) was observed within 30 min of hypoxic challenge which remained upregulated at the same level even after 4 hrs of hypoxia. VEGF receptors, Flk-1 and Flt-1 were also found to be upregulated by hypoxic preconditioning (Fig. 4). A modern experimental strategy for treating myocardial ischemia is to induce neovascularization of the heart by use of “angiogens”, mediators that induce the formation of blood vessels, or angiogenesis [19, 20].

Angiogenic factors

VEGF-System

The process of angiogenesis is regulated by the signals obtained from the transmembrane receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (Src family) of endothelial cells. Flk-1 and Flt-1 are two such RTKs , which together with their ligand VEGF, have been shown to control blood vessel development during embryogenesis [21,22]. This receptor/ligand system has been shown to augment neovascularization [23-26]. VEGF is not only an endothelial cell-specific angiogenic factor but also a critical regulator of angiogenesis that stimulates proliferation, migration, and proteolytic activity of endothelial cells [27]. Yet the signaling pathways that modulate the mitogenic effects of VEGF in vascular endothelial cells are still ill defined [28]. A recent study demonstrated VEGF localization and expression in the embryonic/fetal heart and remained high during the early postnatal period when capillary proliferation is high [29].

ANG-Tie system

The Tie receptors, Tie-1 and Tie-2, are among many receptor tyrosine kinases expressed on endothelial cells [30]. These unique RTKs have received great attention for their possible function in angiogenesis [31-35]. The multiple gene family motifs that comprise the Tie RTKs has led to the notion that Tie-1 and Tie-2 may play a role in hematopoietic cell differentiation and/or in blood endothelial cell interactions [36,37]. Recently, the ligands of the Tie-2 receptor have been identified as Ang-1 and Ang-2, also known as angiopoietins. The name angiopoietin reflects the role of this protein in angiogenesis [38] and its potential role in hematopoiesis. Ang-1 is the major physiological ligand for Tie-2 which is responsible for recruiting and sustaining periendothelial support cells [39]. Ang-2 is found to be responsible in disrupting vessel formation in the developing embryo by antagonizing the effects of Ang-1 and Tie-2.

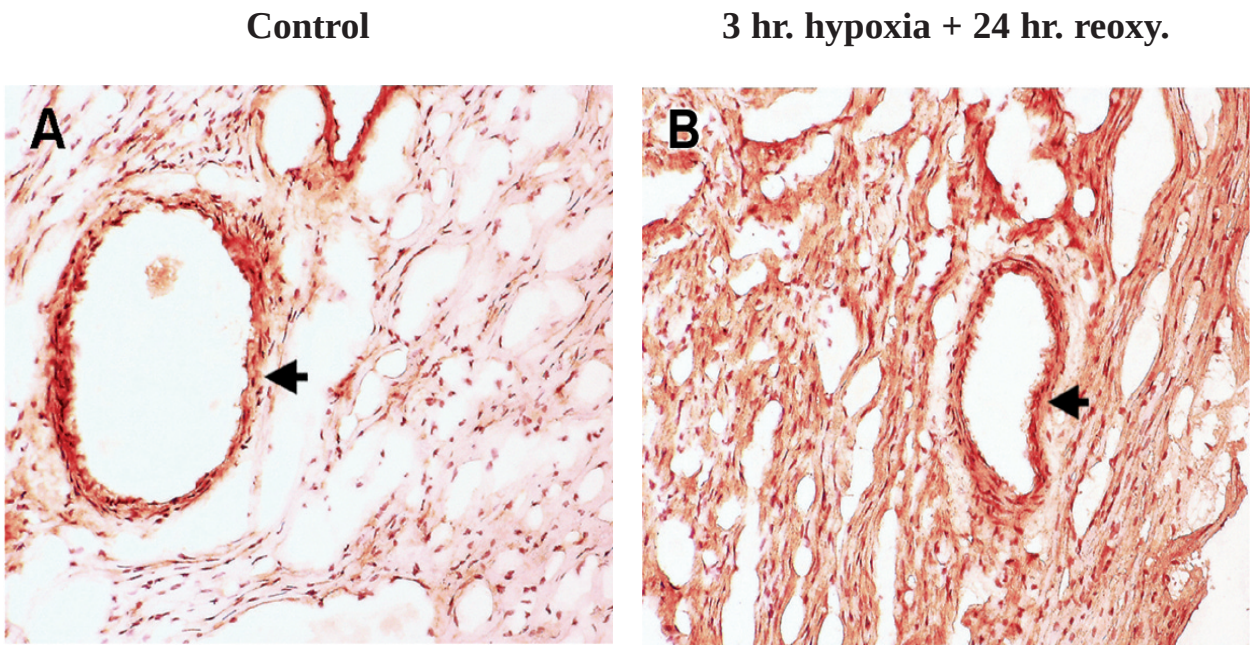

Fig. 3 Immunohistochemistry for *VEGF* Representative sections of rat ventricular myocardium immunostained for *VEGF*.

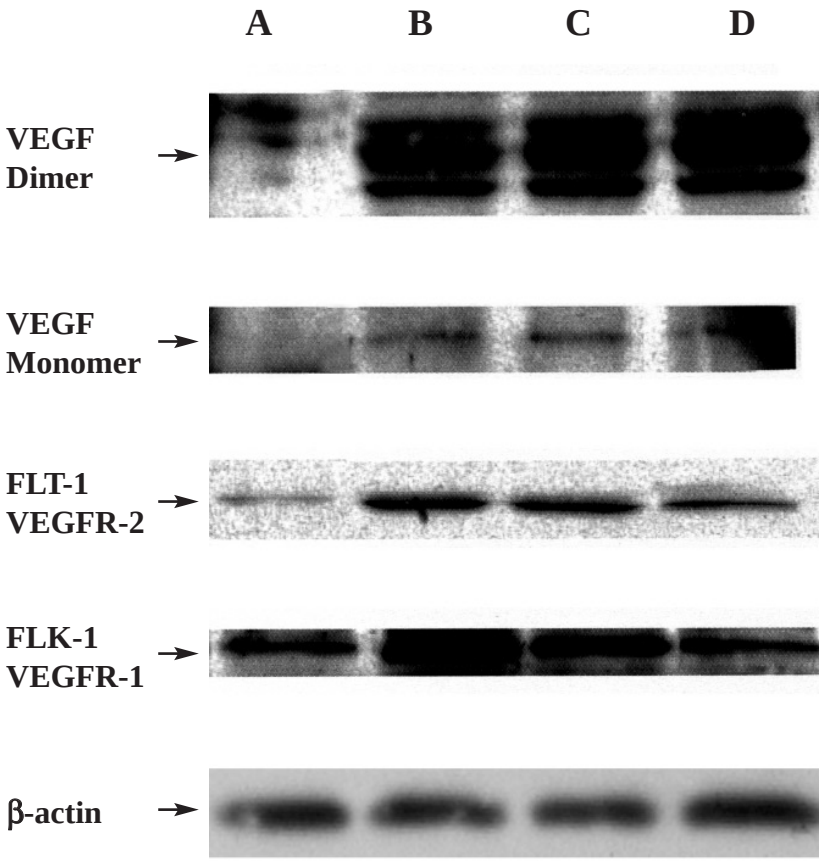

Fig. 4 Representative western blots showing time course of the early effects of systemic hypoxia on expression of *VEGF, Flk-1 and Flt-1* in rat myocardium *in vivo*. VEGF monomer proteins were expressed as 40 kDa and 20 kDa bands whereas Flk-1 and Flt-1 proteins were expressed as 150 kDa and 200 kDa respectively. Similar results were obtained in six independent experiments performed in triplicate. Densitometric scanning of blots were used to determine levels of proteins relative to baseline control (Lane 1). Lane 1: Baseline; Lane 2: 60 min of hypoxia; Lane 3: 2 hrs of hypoxia; Lane 4: 4 hrs of hypoxia. All the animals after hypoxia were exposed to 24 hrs of reoxygenation.

Therefore Ang-2 represents a natural Ang-1/Tie-2 inhibitor. Several reports have already established the involvement of Ang-1 in the maturation and stabilization of developing neovasculature [38] whereas Ang-2 may cause destabilization required for additional sprout formation [40]. Tie-1 and Tie-2 are homologous to each other, but unlike the VEGF receptors, they contain matrix association motifs in their extracellular domains. Both are expressed very early in development [41]. Tie-2 is expressed in the blood islands and in intraembryonic angioblasts, where it appears earlier than von Willebrand factor.

Capillary growth is known to be a rapid process and is promoted by the synergestic effect of βFGF and VEGF. Most recently it was demonstrated that in a rat model of myocardial regional ischemia-reperfusion, LPS pretreatment reduced infarct size and this protection against myocardial infarction was preceded by cardiac expression of βFGF and VEGF, and accompanied by an increase in myocardial capillary density. It was also shown by double immunofluorescent examination of myocardial microvascular density that myocardial capillary density was significantly increased after 3 days of LPS treatment [42]. It is known that inflammation , like ischemia and hypoxia, induces the expression of angiogenic growth factors, specifically bFGF and VEGF, leading to microvasculature growth [43-45]. It is demonstrated that LPS administration increases both circulating and myocardial levels of the proinflammatory cytokine TNF-α [46]. There are several reports which suggests TNF-α as a pro-angiogenic agent [47-49].

Recent studies have demonstrated that administration of βFGF or VEGF to the heart can protect ischemic myocardium by the induction of angiogenesis [50-52]. In another study it was shown that addition of a high concentration of VEGF to hyperkalemic cardioplegia solution improved functional recovery after 120 min of hypothermic global ischemia in the isolated rat heart model. This finding establishes that growth factor, VEGF has a direct cardioprotective effect [53]. Among various triggers of angiogenesis, tissue hypoxia has been identified as being a particularly important stimulus for the induction of new vessel growth [54]. Tissue hypoxia exerts pro-angiogenic action through various angiogenic factors, the most notable being vascular endothelial growth factor which has been chiefly associated with initiating the process of angiogenesis through the recruitment and proliferation of endothelial cells. Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2) are endothelial specific tyrosine kinase receptors of VEGF through which its effects are primarily mediated [55,56]. The fact that VEGF, Flk-1 and Flt-1 expressions are upregulated in response to hypoxia *in vitro* and *in vivo* [57-60] and to ischemia *in vivo* [61-63] is well established, although there are conflicting reports with regard to Flk-1 *in vitro*, suggesting the involvement of adenosine acting as a paracrine mediator through the A_2 receptor [64,65]. The biological functions of VEGF, triggered by external stimuli, are initiated through the activation of intracellular signal transduction cascades involving specific kinases. It is reported that a rapid increase in VEGF expression under hypoxic challenge is due to the presence of hypoxia–inducible factor (HIF) sensitive elements located in the VEGF promoter which upregulated the transcription factor of VEGF [66]. Furthermore, endothelial cells detect external angiogenic stimuli via oncogenes [67].

Regulation of angiogenic factors by MAP kinases

Mitogen activated protein kinase (MAPK) activation is found in cells exposed to mitogens, including bFGF (basic fibroblast growth factor) [68]. In an another study it was shown that the reduced activation of MAPK by antisense expression blocks the proliferative action of bFGF in fibroblasts [69]. Recently, it was reported that VEGF stimulated phosphorylation of MAPK in rat liver sinusoidal endothelial cells [70]. Another study demonstrated the ability of VEGF to upregulate Ang-2 through its Flk-1 receptor via the PKC and MAPkinase pathway [71]. There are several members of the PKC protein family. However, PKC -a and -b are not observed in adult myocytes, but PKC-e, -d, -z are present in detectable amounts [72]. Whether PKC-e, -d, -z have any role in angiogenesis is a relevant unanswered question. Inhibitors of PKC are available [73], and this may allow such an analysis. Most of the studies are done in vitro to show the

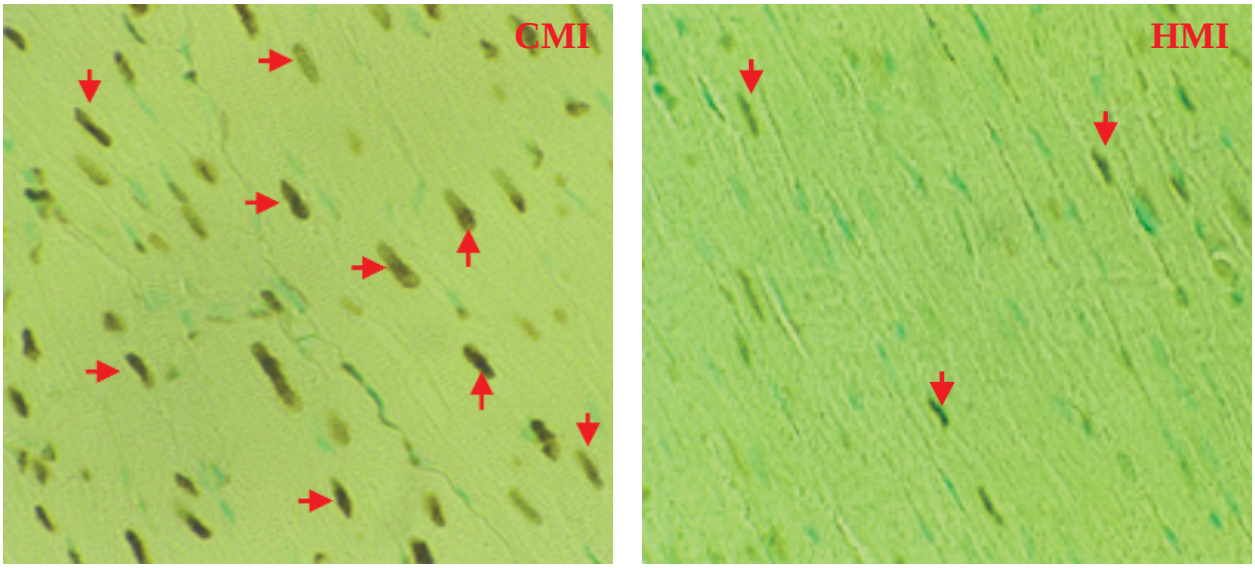

Fig. 5 TUNEL assay for apoptotic cardiomyocytes (brown in colour) were performed (after 2 days of LAD occlusion on confocal laser microscopy. Representative photomicrographs showing immunohistochemical staining of extended DNA (shown by arrows). Where CMI= Control, myocardial infarction (MI), HMI= Hypoxic preconditioned group followed by LAD occlusion. Magnification x1200.

molecular regulation of angiogenic factors by MAPkinases. The role of PKC and MAPkinases in the myocardial angiogenesis in vivo is yet to be established.

Involvement of transcription factors in the process of angiogenesis

Several transcription factors, including HIF-1 [74], AP-1 [75], and NFκB [76], are known to be regulated by hypoxia. The activated form of NFkB is a heterodimer which consists of two proteins, a p65 subunit and a p50 subunit. In normal cells NFκB is maintained in the cytoplasm by protein-protein interaction with inhibitor IκB. Recently it was demonstrated in mice model that NFκB activation is obligatory for retinal angiogenesis and it was also documented that the administration of pyrrolidine dithiocarbamate (PDTC) suppressed retinal neovascularization [77]. In another study, it was documented that hypoxia/reoxygenation, and not hypoxia alone, can cause formation of reactive oxygen species (ROS) and the activation of the NFκB both of which were inhibited by ROS-scavengers, and was accompanied by inhibition of tube formation in angiogenesis. Therefore, in clinical setting of hypoxia/reoxygenation during ischemic pre-conditioning, the activation of ROS-dependent intracellular signaling may accelerate the rate of neovascularization also *in vivo* [78]. Hypoxia has been shown to induce NFkB activation and increased IL-8 as well as VEGF gene expression in glial cells *in vitro*. Furthermore, PDTC, a very specific inhibitor of NFkB activation, prevented the induction of IL-8 gene expression, but had no effect on the VEGF gene in *in vitro* study. This finding suggested that IL-8 gene is induced by hypoxia and mediated by NFkB may contribute to the pathogenesis of intraoccular neovascularization [79].

The AP-1 binding complex consists of either Jun-Fos heterodimers or Jun-Jun homodimers [80]. Several studies have shown that AP-1 and NFkB are differentially activated by oxygen tension. Several potential binding sites for the transcription factors AP-1, AP-2, and SP-1 are localized in the VEGF gene promoter [81]. TNFα or bFGF appears to stimulate expression of the VEGF gene through SP-1 on its promoter [82]. Among eight human glioma cell lines, cellular mRNA levels of transcription factors SP-1 and AP-1 were found to be closely correlated with those of VEGF [83].

VEGF mediated angiogenesis is associated with enhanced cell survival

Recent report suggested VEGF-induced expression of Bcl-2 which eventually functions to enhance the survival of endothelial cells in the toxic, oxygen-deficient environment [84]. This report points out that enhanced level of VEGF may have some role in the inhibition of endothelial cell apoptosis. In our hypoxic preconditioned rat myocardial model, we have found cardiomyocyte apoptosis to be inversely proportional to VEGF expression [85] (Fig. 5). Another very recent investigation was that VEGF a potent promoter of angiogenesis, upregulates the expression of the intracellular adhesion molecule-1 (ICAM-1) through a novel pathway that includes phosphatidylinositol 3 OH-Kinase (PI3K) and AKT resulting in the migration of brain microvascular endothelial cells. It was found that in vitro VEGF treatment phosphorylates AKT in a PI3K-dependent manner [86]. The PI3K/AKT pathway appears to be a general mediator of cytokine induced survival and anti-apoptotic signals. Recently, pro-apoptotic factor, BAD, was reported to be phosphorylated by activated AKT on a serine residue causing BAD to dissociate from BCL-X_L. No *in vivo* study have been done so far to investigate the involvement of PI3K/AKT pathway and endothelial cell survival. Another recent study reported inhibition of endothelial cell apoptosis by Ang-1 via the Akt/survivin pathway which contributed Ang-1 mediated stabilization of vascular structures during angiogenesis [87]. It is also reported that activation of the MAPK pathway together with inhibition of SAPK/JNK activity by VEGF appears to be a key event in determining whether an endothelial cell is going to survive or will undergo programmed cell death. It is clear from the above discussion that inhibition of apoptosis may represent a major aspect of the regulatory activity of VEGF on the vascular endothelium for angiogenesis.

Summary & conclusion

In summary, the above discussion should make it clear that apart from rendering the myocardium tolerant to ischemic reperfusion injury, preconditioning also potentiates angiogenic response. Recently, our study demonstrated for the first time that both ischemic and hypoxic preconditioning triggered myocardial angiogenesis at the capillary and arteriolar levels which nicely corroborated with the improved myocardial contractile function. Hypoxic preconditioning resulted in the stimulation of VEGF, the most potent angiogenic factor known to date. In concert, endothelial cell specific tyrosine kinase receptors, Tie 1, Tie 2 an Flt-1 and Flk-1 were also significantly enhanced in the preconditioned myocardium. The redox-regulated transcription factor NFkB was found to play an essential role in the preconditioning regulation of angiogenesis.

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