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Review Article

# Vascular Endothelial Function in Ischemia/Reperfusion Injury: Pathophysiological Mechanisms and Clinical Implications for Postischemic Myocardial Protection

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## Abstract

Ischemia/reperfusion (I/R) injury still remains a major challenge in standard medical treatments of ischemic heart disease, i.e., thrombolytic therapy and primary percutaneous coronary intervention as well as in open heart surgery. Development of cardio protective strategies against I/R injury is of great clinical importance. Vascular endothelial dysfunction plays a significant role in myocardial I/R injury, which makes endothelium an attractive target for postischemic myocardial protection. Recent advances in understanding the molecular mechanisms underlying endothelial I/R injury laid the foundation for future development of novel strategies targeting endothelium for prevention and/or treatment of myocardial I/R injury. This review summarized the pathophysiological mechanisms of endothelial I/R injury and discussed the significance and potential of endothelium targeting strategies in postischemic myocardial protection.

**Keywords:** Endothelium; Gap junction; Ischemia/Reperfusion; Nitric oxide; Ion channels

## Abbreviations

Cx: Connexin; EDHF: Endothelium-derived hyperpolarizing factor; ENOS: Endothelial nitric oxide synthase; EPC: Endothelial progenitor cell; EETs: Epoxyeicosatrienoic acids; H/R: Hypoxia/reoxygenation; ICAM: Intercellular adhesion molecule; IKCa: Intermediate conductance Ca2+-activated K+ channel; I/R: Ischemia/reperfusion; BKCa: Large-conductance Ca2+-activated K+ channel; NO: Nitric oxide; NF-κB: Nuclear factor kappa-B; ONOO-: Peroxynitrite; ROS: Reactive oxygen species; sEH: Soluble epoxide hydrolase; Soluble epoxide hydrolase; SKCa: Small conductance Ca2+-activated K+ channel; TRP channel: Transient receptor potential channel; VCAM-1: Vascular cell adhesion molecule-1

## Introduction

The vascular endothelium is a single layer of cells that lines the entire circulatory system. By counteracting leukocyte adhesion and platelet aggregation to prevent inflammation and thrombosis and actively regulating vascular tone with the production of vasoactive substances, endothelial cells play a key role in maintaining vascular health. Disturbance of functional integrity of endothelium, known as "endothelial dysfunction", represents a complex pathophysiological entity including inflammatory activation and perturbation of anticoagulatory properties as well as abnormal vasomotion [1]. Endothelial dysfunction significantly contributes to the pathogenesis of a variety of cardiovascular disorders including myocardial ischemia [2]. Ischemic heart disease is the most common cause of myocardial ischemia. Previous studies have demonstrated the pivotal role of endothelial dysfunction in the initiation and progression of this disease [3,4]. Moreover, strong associations have been reported between endothelial dysfunction and a number of well-defined risk factors for ischemic heart disease such as smoking, hypertension, obesity, and diabetes [5]. Myocardial ischemia is inevitable in cardiac surgery requiring cardiopulmonary bypass. The no- or low-reflow phenomenon after myocardial ischemia/reperfusion (I/R) resulting from endothelial edema, neutrophil and platelet plugging, microthrombosis, and enhanced vasomotor may lead to inadequate coronary perfusion that further compromises cardiac function [6].

## Pathophysiological mechanisms of endothelial dysfunction in myocardial I/R

I/R induces vascular endothelial dysfunction through multiple mechanisms including cytotoxicity caused by pH change, oxidative stress resulting from overproduction of Reactive Oxygen Species (ROS), and Endothelial Nitric Oxide Synthase (eNOS)-Nitric Oxide (NO) inhibition, etc. [7,8]. Studies in recent years provided new insights into the molecular mechanisms of endothelial I/R injury such as modulation of ion channels and gap junction proteins. The role of acidosis-induced cytotoxicity in ischemic endothelial damage was evidenced by ischemic acidosis-induced activation of caspases, i.e., caspase-12 and caspase-3, in endothelial cells of coronary arteries [9]. By up regulation of the anti apoptotic protein Bcl-xL, acidic preconditioning protects coronary endothelial cells from ischemic apoptosis [10]. In addition, extracellular acidosis strongly suppresses Ca2+ entry into endothelial cells thereby inhibiting the production of vasoactive substances, which may also be involved in I/R-induced endothelial dysfunction [11]. ROS is abundantly generated by cardiomyocytes, coronary vascular endothelium, and inflammatory cells during I/R through incomplete reduction of O2 in which xanthine oxidase, NADPH oxidase, NO synthase (unconjugated), cyclooxygenase, and lipoxygenase may all be involved [8]. Activation of endothelial cell by oxidative stress promotes intravascular




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microthrombosis, reduction of blood flow and activation of inflammatory cells. Expressions of E-selectin, P-selectin, and intercellular adhesion molecules (ICAMs) on the surface of activated endothelial cells promote the recruitment of neutrophils, the principal effector cells of inflammation during I/R [12]. Nuclear factor kappa-B (NF-κB) plays a key role in I/R-induced endothelial cell activation. Tyrosine phosphorylation of IκBκ induced by oxidative stress results in the dissociation of this inhibitory protein from NF-κB, leading to the nuclear translocation of NF-κB and subsequent activation of transcription of proinflammatory, procoagulant, and vasoactive genes expressed in endothelial cells, which consequently initiates and propagates myocardial I/R injury [13]. In addition to be a target of ROS, endothelial cells are also an important source of ROS. ROS generated by endothelial cells through xanthine oxidase, NADH/NADPH oxidase, and uncoupled eNOS significantly contributes to vascular dysfunction after I/R that involves acceleration of NO inactivation [14].

Endothelial permeability increases following myocardial I/R. The loss of barrier function of endothelial cells can be attributed to ROS released from activated leukocytes that cause changes in endothelial cytoskeletal structures and promote the formation of intercellular gap [15]. Activation of endothelial contractile machinery due to cell re-energization as well contributes to endothelial barrier failure [16]. Endothelial barrier dysfunction consequently promotes migration of neutrophils and other inflammatory cells into the injured myocardial tissue and further potentiates I/R injury. Moreover, I/R disrupts the balance between endothelium-derived constricting and relaxing factors thus interrupts blood flow and organ perfusion. I/R increases the production of vasoconstrictors such as endothelin-1 [17]. A considerable body of evidence suggests the significance of reduction of endothelium-derived relaxing factors, in particular, NO and Endothelium-Derived Hyperpolarizing Factor (EDHF) in the disturbance of blood flow in myocardial ischemia and related conditions [18-23].

In addition to its potent vasodilatory effect, NO inhibits platelet aggregation and leukocyte adhesion as well as vascular smooth muscle proliferation to act as an important component of the endogenous defense mechanism against vascular injury, inflammation, and thrombosis. The decrease of NO bioavailability is a well known consequence of myocardial I/R. Multiple mechanisms including eNOS inhibition [24,25], arginase activation [26,27], and increased production of ROS [28] are involved in I/R-induced NO loss through reduction of production and/or acceleration of inactivation. Inhibition of store-operated Ca2+ entry by acidosis results in decreased production of NO, which may also contribute to endothelial dysfunction during ischemic assault [11]. In fact, in an in vitro I/R model, measurement of NO by using a NO micro sensor provided a direct evidence of the decrease of NO in coronary arteries after hypoxia/reoxygenation (H/R) exposure [29]. Uncoupling of eNOS is another mechanism by which myocardial I/R compromises eNOS-NO function. In stead of producing NO, uncoupled eNOS becomes a source of ROS generation [30]. This functional switch of eNOS occurs when substrate L-arginine or cofactor tetrahydrobiopterin (BH4) is insufficient, which in myocardial I/R can result from arginase activation that increases the consumption of L-arginine, and ROS production (particularly peroxynitrite ONOO-) that leads to oxidization and degradation of BH4 [31]. Reduction of NO and production of O2.- worsen endothelial I/R injury. Moreover, NO and O2.- can affect primary contractility of actin-myosin fibers within myocytes, putatively via effects on Ca2+ storage in the sarcoplasmic reticulum. Diminished myofiber contraction resulting from NO inhibition and O2.- overproduction significantly affects cardiac output [32]. Contribution of EDHF in vasodilatation increases as vessel size decreases [33,34], which highlights the significance of EDHF in blood flow regulation. Opening of intermediate and small conductance Ca2+-activated K+ channels (IKCa and SKCa) on the plasma membrane of endothelial cells underlies the classical EDHF pathway [35]. IKCa and SKCa opening induces endothelial membrane hyperpolarization that can be conducted along the endothelium via homocellular endothelial gap junctions and transmitted to smooth muscle cells through myoendothelial gap junctions to cause vasodilatation. IKCa and SKCa activation may also induce K+ efflux from endothelial cells to elicit hyperpolarization and relaxation of adjacent smooth muscle cells by activating inwardly rectifying K+ (Kir) channels and Na+-K+-ATPase on the smooth muscle membrane [36]. In some vasculature including coronary arteries, non-classical EDHF response mediated by epoxyeicosatrienoic acids (EETs) may also exist. EETs not only activate endothelial IKCa and SKCa but also open myocyte large-conductance KCa (BKCa) to relax vessels [37]. Investigations in the past decade revealed the impact of I/R on EDHF-mediated endothelial function. Although potentiation of the EDHF-type response was reported in animal models of myocardial I/R and cerebral I/R [38,39], which supports the “compensatory or backup” theory of EDHF-mechanism in conditions involving NO loss, contradictory evidence is also available that shows the impairment of EDHF-mediated function under I/R conditions. For example, in porcine coronary arteries exposed to H/R, the EDHF-mediated relaxation was significantly attenuated [22,40,41]. H/R also blunted the EDHF-response in coronary microveins [23]. Further membrane potential measurement showed a decrease of hyperpolarization mediated by EDHF in smooth muscle cells of coronary vasculature [42].

Furthermore, we recently demonstrated that H/R inhibits IKCa and SKCa currents in coronary endothelial cells and the inhibition of IKCa and SKCa activity underlies the impairment of EDHF responses caused by H/R [42].

## Significance/Potential of endothelial protective strategies in myocardial I/R injury

To date, I/R injury still remains a major challenge in standard medical treatments of ischemic heart disease, i.e., thrombolytic therapy and primary percutaneous coronary intervention [43], and in open heart surgery. Myocardial I/R induces coronary endothelial dysfunction that in turn promotes myocardial injury. Exaggerated inflammatory reactions following endothelial cell activation are closely associated with oxidative stress during myocardial ischemic assault, which rationalizes the traditional anti-inflammatory and antioxidant strategies for endothelial and myocardial protection. Postischemic cardiac performance may benefit from well-preserved coronary blood flow by strategies protecting endothelial dilatory function, i.e., NO and EDHF pathways. New approaches targeting cellular mechanisms underlying these endothelium-derived relaxing factors have the potential to become new treatments for myocardial ischemia.

## Anti-inflammation and antioxidant strategies for cardioprotection

Cardioprotection conferred by interventions targeting neutrophil influx, such as neutralization of P-selectin or depletion of neutrophil has been reported in ischemic myocardial injury [44,45]. Administration of monoclonal antibody against leukocyte adhesion molecule CD18 (ligand for ICAM-1) protects coronary endothelium and myocardium in neonatal lamb hearts following cardioplegic arrest, evidenced by preserved coronary blood flow and better recovery of left ventricular developed pressure [46]. Inflammatory reactions resulting from endothelial cell activation can be suppressed by NF-κB inhibition. Transfection of NF-κB decoy oligonucleotides into isolated rat heart blocked ICAM-1 up regulation and inhibited neutrophil adhesion to small coronary venules [47]. The dramatic increase of NF-κB in patients undergoing heart surgery with cardioplegic intervention [48] added clinical evidence supporting the potential of NF-κB inhibition in postischemic myocardial protection.

It has to be mentioned that although cardioprotective effect of anti-inflammatory strategies has been shown in a number of animal experimental studies, clinical trials aiming to inhibit inflammation however yielded unsatisfactory results, suggesting that inflammation is not solely an injurious process, but also mediates processes essential for proper tissue healing. Therefore, balancing the inflammatory forces between damage and repair needs to be emphasized in future development of anti-inflammatory strategies, such as strategy targeting endothelial cell activation, for cardioprotection against I/R injury. Endothelium-dependent vasodilator responses of coronary arteries were better preserved after cardiac arrest using cardioplegic solution containing inhibitors of hydroxyl radical synthesis, i.e., deferoxamine or manganese superoxide dismutase [49]. Inclusion of organic antioxidants such as ascorbate and deferoxamine in St Thomas' Hospital cardioplegic solution improved the recovery of aortic flow in rat heart after global ischemic arrest [50]. The protective effect of antioxidants on endothelium involves the inhibition of ROS-induced endothelial cell activation and NO inactivation [51,52]. As the role of enzyme sources of endothelium-derived ROS become clear, it is possible to develop more specific therapies targeting endothelial redox mechanisms for myocardial protection.

## Significance of targeting eNOS-NO mechanism in cardioprotection

The significance of NO in inhibiting neutrophil accumulation, inactivating superoxide radicals, and improving coronary blood flow establishes the role of this intracellular signaling molecule in myocardial protection. Moreover, NO was found to mediate the cardioprotective effect of a number of clinically used strategies such as preconditioning and postconditioning [53], which further supports the concept of targeting eNOS-NO mechanism for myocardial protection under I/R conditions.

Early attempts to enhance NO function include application of NO precursor L-arginine or NO donors such as nitroglycerin. Administration of these agents or supplementation in cardioplegia preserves postischemic endothelial function in both animals and humans and improves postischemic ventricular performance [54-58]. In fact, the use of NO-donor drugs is considered an effective replacement therapy in "NO-deficient" disorders. However, the reduced responsiveness to nitrovasodilators, caused by nitrate resistance and nitrate tolerance, yet remains a problem to be solved. Strategies targeting mechanisms by which I/R inhibits NO function were further developed, including inhibition of arginase activation [27], restoration of eNOS down-regulation [25], and modulation of eNOS uncoupling [59]. Use of arginase inhibitor restored the NO-mediated function in I/R vessels [27]. Addition of eNOS-transcription enhancer AVE3085 in St. Thomas' Hospital cardioplegia was observed to restore NO production suppressed by H/R and protect coronary dilator responses [25]. Experimental studies in cultured bovine aortic endothelial cells demonstrated that exogenous BH4 supplementation during oxidative assault prevents eNOS uncoupling and increases NO production [31]. Further, in a co-culture system of cardiomyocytes and endothelial cells, increasing BH4 content in endothelial cells by either pharmacological or genetic approaches was able to reduce the susceptibility of cardiomyocytes to H/R injury [60]. Recent studies demonstrated that human eNOS gene is subject to alternative splicing and the expression of splice variants, i.e., eNOS13A, produce truncated proteins lacking the reductase domain with no eNOS activity. Moreover, eNOS13A forms heterodimers with full-length eNOS and such heterodimerization significantly reduces eNOS activity [61,62]. These findings suggested that regulation of eNOS activity via modulation of the expression of eNOS isoforms could be of potential therapeutic interest in cardiovascular disorders including myocardial I/R injury in which endothelial dysfunction plays a role in the pathogenesis.

## Cardioprotective potential of EDHF preservation

Preservation of EDHF component can be achieved by several approaches that have been proven effective in experimental studies. Addition of EET11,12, a possible chemical analogue of EDHF to cardioplegic solutions protects endothelial function of coronary arteries with restoration of EDHF-mediated responses [63,64], which can be explained by the direct "EDHF mimetic" effect of EET11,12. Interestingly, a recent study in an in vivo rat model of infarction demonstrated that administration of EETs prior to ischemia activates eNOS and increases NO production [65], which provided a new insight into cardioprotective mechanisms of EETs [66]. In addition to exogenous administration of EET analogs, approaches aiming to increase the endogenous concentration of EETs also show therapeutic potential in myocardial ischemia that include inhibition of soluble epoxide hydrolase (sEH) [67] to suppress EETs metabolism and of cytochrome P450 epoxygenases to increase EETs production [68].

## Cardioprotective potential of targeting gap junctions

Gap junctions formed by connexins (Cx) play an important role in cell¬-cell communication and homeostasis in various tissues including vasculature, which enable a direct passage of ions, metabolites, or electrical signals from one cell to another. Electrical coupling along the endothelium and between endothelium and smooth muscle is central in arteriolar conducted response and control of vascular resistance. The vascular gap junctions are assembled from one or more of four connexin proteins: Cx37, Cx40, Cx43, and Cx45. Cx40 and Cx43 are expressed in both endothelial and smooth muscle cells while Cx37 is typically confined to endothelium and Cx45 locates at smooth muscle [69]. Endothelial expression of Cx40 is influenced by various factors such as oxidative stress, pro-thrombotic molecules, pro-inflammatory cytokines, and classical cardiovascular risk factors [70,71]. A recent study in a clinically relevant setting of I/R injury showed that the expression of Cx40 disappears from the endothelium in the infarct zone and in mice with endothelial-specific deletion of Cx40, infarct size increases after I/R. The cardioprotective effect of endothelial Cx40 in cardiac I/R injury was suggested to be associated with a decrease in neutrophil infiltration through ecto-5′-nucleotidase/CD73-dependent regulation of Vascular Cell Adhesion Molecule-1 (VCAM-1) expression at the surface of endothelial cells [72-74]. Consistently, in a hind limb ischemic model, Cx40 deficient animals exhibited profound and rapid failure of ischemic limb survival [75]. Studies in a monolayer of cultured microvascular endothelial cells showed that hypoxia followed by abrupt reoxygenation reduces interendothelial electrical coupling via oxidant- and PKA-dependent signaling that targets Cx40, which provided a mechanistic explanation for the compromised arteriolar function following H/R [76]. Considering that eNOS and Cx40 can exist in a complex and endothelial Cx40 expression is essential for proper expression and function of eNOS [77,78], I/R / H/R-induced Cx40 modulations are therefore expected to result in functional changes of eNOS-NO pathway. Direct electrical communication between endothelial and smooth muscle cells via myoendothelial gap junctions plays an essential role in EDHF signaling, which further reveals the relevance of connexin proteins to the endothelial control of vascular tone. Blockade of myoendothelial gap junctions with mimetic peptides specifically against Cx37, Cx40 and Cx43 has been observed to prevent endothelium-dependent subintimal smooth muscle hyperpolarization [79,80]. Rapid endothelial cell-selective loading of Cx40 antibody also blocked EDHF-type signaling [81]. Given the important role of gap junctions in conducting vasodilator responses, manipulation of connexin function and/or expression may represent a potential approach for tackling endothelial dysfunction. The improvement of vasorelaxation in response to preconditioning was demonstrated to be associated with increases of Cx40 and Cx43, as well as a more efficient gap junction coupling in endothelial cells [82]. However, successful translation of these basic scientific discoveries into clinical application will require further studies and future developments of selective pharmacological tools that allow targeting gap junctions in a connexin-isoform and cell type-specific manner.

### Cardioprotective potential of targeting endothelial ion channels

Endothelial ion channels, in particular, Ca2+-permeable channels, i.e, Transient Receptor Potential (TRP) channels [83], and K+ channels, i.e, IKCa and SKCa, emerge as promising therapeutic targets for endothelial I/R injury. An increase of [Ca2+]i in endothelial cells is required for the activation of NO generating enzyme eNOS [84]. Opening of IKCa and SKCa or/and production of EETs that underlie the EDHF action also depend on endothelial [Ca2+]i rise [35,85]. On the other hand, membrane hyperpolarization of endothelial cells resulting from IKCa and SKCa opening in turn enhances driving force for Ca2+ entry, promoting Ca2+ influx and NO production [86]. These lines of evidence suggest the significance of Ca2+-permeable and K+ channels and the functional interplay between these two distinct types of channels in the modulation of endothelial function.

IKCa/SKCa and TRP channels were found to be affected by I/R and hyperkalemic exposure, which provided scientific basis for targeting these channels during cardiac surgery for endothelial protection. In coronary arteries exposed to H/R, pharmacological activation of IKCa and SKCa channels improves EDHF-responses including relaxation and hyperpolarization [42]. The potential of IKCa/SKCa activators in the treatment of cardiovascular disorders through the improvement of endothelium-derived hyperpolarizations and NO-mediated function was discussed in depth in recent review articles [87,88].

I-R/H-R affects TRP channels, i.e, TRPC3 and TRPV4, and associated vascular endothelial function. Through inhibiting membrane translocation of the channel, H/R suppresses TRPC3 channel activity and Ca2+ influx via TRPC3 in coronary endothelial cells, resulting in reduction of NO production. Activation of TRPC3 channels restores NO production in coronary arteries subjected to H/R [29]. Most recently, we demonstrated that supplementation of the TRPC3 channel activator in hyperkalemic cardioplegia such as St Thomas' Hospital and Histidine-Tryptophan-Ketoglutarate solutions preserves TRPC3-mediated Ca2+ influx in endothelial cells and improves EDHF-mediated relaxation of coronary arteries [89]. In a mice model of prolonged hypoxia and reoxygenation, amplification of EDHF-mediated relaxation induced by preconditioning is associated with an increase of TRPV4 expression in endothelial cells. Preconditioning also increases eNOS phosphorylation to provide cardioprotection through a TRPV4-dependent mechanism [82]. These findings laid the foundation for future development of endothelial TRP channel-targeting strategies for postischemic myocardial protection.

### Cardioprotective potential of cell-based therapy

It was reported that endothelial function in humans is associated with the number of circulating endothelial progenitor cells (EPCs) [90]. Increases in number of EPCs and NO production mediate the endothelial protection conferred by ischemic preconditioning in humans [91]. Intracoronary delivery of progenitor cells in patients with chronically occluded coronary arteries led to improvement in coronary flow reserve and cardiac function at 3-months post transplant [92]. A recent study showed that the EPC-driven postischemic myocardial protection is partially mediated by activation of the VEGF-PI3K/Akt-eNOS pathway [93]. MacArthur and colleagues recently developed a hydrogel delivery system enabling the sustained release of a bioactive EPC chemokine, which induces continuous homing of EPCs and effectively improves left ventricular function in a rat model of myocardial infarction [94]. However, one must admit that knowledge of progenitor cells still remains inadequate and more preclinical and clinical studies are needed.

## Conclusion

In summary, the significance of vascular endothelial dysfunction in myocardial I/R injury makes endothelium an attractive therapeutic target for postischemic myocardial protection. Development of approaches controlling endothelial cell activation and more specific interventions targeting endothelial redox mechanisms will help alleviate myocardial injury following I/R. Endothelial progenitor cells represent an emerging cell-based strategy for promoting vascular repair and restoring microvascular perfusion of ischemic myocardium. Moreover, new insights into molecular mechanisms of endothelial dysfunction in relation to NO and EDHF during I/R and cardioplegic intervention, such as connexin proteins and ion channels, may lead to novel therapeutic strategies with the potential to improve prognosis of myocardial ischemia. The great hope of these endothelium targeting strategies for postischemic myocardial protection remains to be realized with further preclinical and clinical research.

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