Role of Endothelial Cells in Myocardial Ischemia-Reperfusion Injury

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Abstract: Minimizing myocardial ischemia-reperfusion injury has broad clinical implications and is a critical mediator of cardiac surgical outcomes. "Ischemic injury" results from a restriction in blood supply leading to a mismatch between oxygen supply and demand of a sufficient intensity and/or duration that leads to cell necrosis, whereas ischemia-reperfusion injury occurs when blood supply is restored after a period of ischemia and is usually associated with apoptosis (i.e. programmed cell death). Compared to vascular endothelial cells, cardiac myocytes are more sensitive to ischemic injury and have received the most attention in preventing myocardial ischemia-reperfusion injury. Many comprehensive reviews exist on various aspects of myocardial ischemia-reperfusion injury. The purpose of this review is to examine the role of vascular endothelial cells in myocardial ischemia-reperfusion injury, and to stimulate further research in this exciting and clinically relevant area. Two specific areas that are addressed include: 1) data suggesting that coronary endothelial cells are critical mediators of myocardial dysfunction after ischemia-reperfusion injury; and 2) the involvement of the mitochondrial permeability transition pore in endothelial cell death as a result of an ischemia-reperfusion insult. Elucidating the cellular signaling pathway(s) that leads to endothelial cell injury and/or death in response to ischemia-reperfusion is a key component to developing clinically applicable strategies that might minimize myocardial ischemia-reperfusion injury.

INTRODUCTION

Myocardial ischemia-reperfusion (IR) injury may result from pathological processes such as atherosclerotic coronary artery disease and acute myocardial infarction and/or be secondary to surgical processes such as operating on the arrested heart or cardiac transplantation. In contrast to "ischemic injury" which occurs when oxygen demand exceeds the available blood supply and is associated with cell necrosis, IR injury occurs upon return of blood supply after a period of ischemia and is usually associated with apoptosis (i.e. programmed cell death). When compared to endothelial cells (ECs), cardiomyocytes (CMs) are more sensitive to ischemic injury and have received the most attention in the quest for preventing myocardial IR injury. However, several studies suggest that ECs are more sensitive to IR injury than CMs and that they might be a critical mediator of IR injury in the heart. This review is focused on defining the critical role of the endothelium in myocardial IR injury.

Cardiovascular disease, the number one killer in the United States, is associated with significant short- and long-term mortality and puts significant economic burden on society. In the United States in 2005, more than 16 million people had symptomatic coronary artery disease (CAD), and greater than 1.2 million people are believed to have died from CAD (approximately 1:5.6 deaths). The direct and indirect costs for the care of cardiovascular disease in the United States are estimated to exceed $400 billion in 2008. In the most severe case, the 30-day mortality is still approximately 10%. Overall at one year, mortality for myocardial infarction (MI) is between 5 and 13% [1, 2]. Understanding the basic mechanism(s) of EC injury/death is critical to developing clinically applicable strategies to minimize myocardial ischemia-reperfusion injury.

ECs do more than provide a protective barrier between the lumen and vascular smooth muscle of blood vessels. These dynamic cells contribute importantly to regulating blood flow, neutrophil, platelet and complement activation, and myocardial function [3-9]. Furthermore, ECs are important in modulating the inflammatory response associated with IR injury, including vascular neogenesis [10, 11]. Despite the critical role of ECs in myocardial IR injury, most research has focused on the role of CMs [12-14]. As with all pathological processes, avoiding/minimizing exposure to risk factors is optimal. However, should a coronary event occur, early intervention is more effective than attempting to arrest an ongoing process [11, 15]. With the latter principal in mind, understanding the pivotal role of ECs might lead to the development of more effective therapies that can be applied early in the injury process. The underlying goal of this article is to: (1) review literature on IR induced EC injury and/or death;(2) understand the effects of EC injury on cardiomyo-

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cytes and myocardial function; and (3) discuss potential investigational approaches / strategies.

Ischemia is a process that occurs when the demand for oxygen exceeds the available supply, most commonly as a result of inadequate blood flow. In myocardial infarction (MI), this disruption of flow can result from partial or total occlusion of a large epicardial coronary artery. However, when an occluded vessel is relieved of the blockage, blood flow may not return to pre-occlusion values. In extreme cases, even in the presence of a completely patent vessel, "no-reflow" may occur. "No-reflow" was first described by Kloner in 1974 and was investigated intensely by Menger and Messmer as well as Kloner and colleagues [11, 16-19]. Attenuated flow and / or no-reflow upon reperfusion of an otherwise patent artery might be secondary to a disrupted capillary microcirculation. While many possibilities exist, disruption of the capillary microcirculation distal to the site of occlusion could potentially result from: (1) neutrophil trapping and/or platelet aggregation; (2) myocardial edema-evoked mechanical compression/occlusion of capillaries; and / or (3), swelling and/or damage of ECs in capillaries leading to occlusion of the microcirculation. With regard to the first explanation, while leukocyte and platelet plugging have been demonstrated *in vitro*, this phenomenon has not been demonstrated *in vivo*. Furthermore, "no-reflow" occurs in isolated heart models without leukocytes. Concerning the second explanation, complex models of myocyte edema and interstitial pressure do not completely support that model of myocardial swelling [16, 17]. However, evidence does support a critical role of EC swelling and resultant microcirculatory occlusion in "no-reflow". Specifically, vasodilators such as adenosine, nitroprusside, and verapamil (L-type Ca-channel blocker) are effective at decreasing no-reflow. Additionally, the experimental use of free radical scavengers in isolated striated muscle and heart models is also associated with decreased EC injury and no-reflow [20-22]. Taken together, any and/or all of these proposed explanations could contribute to a greater or lesser extent to attenuated flow / no-reflow that is observed secondary to reperfusion i.e. mechanisms responsible for no-reflow are not precisely known.

"No-reflow" is an extreme example of vascular injury. Intermediate between normal flow and "no-reflow" is reduced flow and decreased vascular reactivity; the latter is the inability of vessels to respond appropriately to an endothelium-dependent and/or –independent vasodilator after an IR

insult [23, 24]. Based on data from isolated vascular ring preparations, arterial relaxation in response to vasodilators such as acetylcholine (an endothelium-dependent vasorelaxing agent) and Nipride (an endothelium-independent vasorelaxing agent) is only mildly decreased following prolonged (four to six hours) periods of ischemia (and no reperfusion) when myocyte damage may precede EC damage [25]. In contrast, much shorter (1.5 hours) periods of ischemia coupled with reperfusion (i.e. IR injury) result in much greater impairment of vascular relaxation [26, 27]. Thus, reperfusion is an important contributor to IR injury (Table **1**).

ECs are more sensitive to IR than to ischemia alone. IR injury is associated not only with immediate vasoconstriction and decreased response to vasodilators at reperfusion but also prolonged vascular dysfunction. In canine infarct models, persistent EC dysfunction can be demonstrated at two but not nine weeks following an IR episode [28]. Although clinical data are more limited, patients suffering from acute MI demonstrate similar vasodilatory dysfunction compared to control patients with chronic atherosclerotic disease. In these patients, early recovery of EC function is associated with improved clinical outcomes [29]. IR injury causes acute and possibly prolonged decreases in vascular function.

Although ECs are less sensitive to ischemic injury, they are quite sensitive to IR injury in the heart [3]. In cell culture, anaerobic glycolytic metabolism can provide adequate energy for ECs [30]. Isolated coronary ECs may catabolize 99% of glucose into lactate [7]. While the physiological relevance of these cell culture data is unclear, they do provide proof of principle that ECs may be more resistant than CMs to ischemia [30]. Given that ECs can tolerate a relatively wide range of oxygen tensions, they have been proposed as biological oxygen sensors [7, 30-32]. Despite being able to withstand long periods of ischemia, reperfusion evokes a variety of cellular abnormalities that occur quickly. After reperfusion, reactive oxygen species (ROS) production increases, nitric oxide (NO) bioavailability is disrupted, an imbalance of Ca^{2+} homeostasis occurs, and cells progress to apoptotic death. Each of these will be discussed.

Consequences of EC ROS Formation

ROS are critical mediators of EC injury. In biological systems, ROS are negatively charged short-lived oxygen species that cause nonspecific and often irreparable oxidative

Table 1.   **Effects of Ischemia and Reperfusion on Endothelium-Dependent Vasorelaxation (% Vascular Relaxation) of Isolated Coronary Arteries from Animal Models [25-27]**

Ischemic Time	Reperfusion Time	% Vascular Relaxation Compared to Baseline
1.5 hrs	-	≈ 85%
1.5 hrs	2.5 min	16±6%
1.5 hrs	3.0 hrs	11± 3%
3.0 hrs	-	63%±6%
4.5 hrs	-	32±4%
6.0 hrs	-	31± 6%

damage. ROS have been implicated in a host of illnesses such as neurodegenerative diseases (e.g. Parkinson's, Alzheimer's), glaucoma, autoimmune diseases (e.g. scleroderma), cancer, hypertension, renal and vascular diseases [33-37]. Under conditions of prolonged extreme ischemia ($FiO_2 \leq 1.5$-3.0%), increased ROS production occurs in human umbilical vein ECs (HUVECs) and this response can be recapitulated in HUVECs after 90-min of ischemia followed by reoxygenation [38, 39]. Major sources of endothelial ROS production include cytosolic xanthine oxidase and complex I and III of the mitochondrial electron transport chain [7, 31, 40]. Overall the evidence suggests that upon an IR insult, the critical site of ROS production is the mitochondria *via* the electron transport chain [30, 40]. The principal ROS produced are O_2^- (superoxide), OH^- (hydroxyl) and $ONOO^-$ (peroxynitrite) anions [23]. ROS production occurs rapidly upon reperfusion and is critical in initiating many downstream events. For example, ROS production appears within minutes of reoxygenation in cultured ECs based on electron paramagnetic resonance (EPR) spectroscopy [41]. Given the short half life of free radicals, *in vivo* data on ROS production are quite limited but ROS formation has been demonstrated in isolated muscle [42] and in rat stroke models [43]. One of the major consequences of ROS production is decreased vasoreactivity [23]. In this regard, ROS production might overwhelm the endogenous antioxidant environment, lower NO bioavailability, and consequently impair endothelium-dependent vasorelaxation. NO, originally known as endothelium derived relaxing factor, is a potent vasodilator [41, 44]. Therefore, NO destruction leads to decreased vasoreactivity. Additionally, the production of ROS leads to the expression of surface adhesion molecules [45, 46] and complement activation [47], which collectively precipitate neutrophil binding to the EC surface. Once at the site of injury, neutrophils are probably a greater source of ongoing ROS production than ECs. In summary, when ECs produce ROS at reperfusion vasoreactivity is decreased *via* mechanisms associated with lowered NO bioavailability together with a cascade of inflammatory events that is initiated after surface adhesion molecules are expressed.

Consequences of Reduced NO Bioavailability

NO is an important modulator of IR injury in experimental models. NO is produced by three NO synthase (NOS) isoforms: inducible NOS (iNOS), endothelial NOS (eNOS) and neuronal NOS (nNOS) [48]. While the primary source of NO in the heart is ECs [7, 24], evidence exists that NOS also is located in cardiac ganglion cells and nerve fibers innervating the sinuatrial and atrioventricular nodes, and local neurons, in addition to the myocardium and coronary/pulmonary vessels [49]. Thus, NO is involved in the neural control of heart rate, myocardial cell function, and neuronal transmission in cardiac ganglia, in addition to its vascular dilatory properties [49].

Endogenous basal NO synthesis is low yet an important determinant of regional and systemic vascular resistance. At the onset of ischemia there is an initial increase in the bioavailability of NO within 5-10 seconds and subsequent vasodilation, but this response tapers within 3-5 minutes [7, 24, 48]. Upon reperfusion NO bioavailability again increases rapidly but wanes within the same time frame as for ischemia [50]. Evidence that the decrease in NO during sustained ischemia and shortly after reperfusion can be prevented by antioxidants implicates ROS-mediated NO destruction [23, 50]. As NO is short lived, it cannot be delivered directly as a therapeutic agent but rather *via* NO donors (e.g. Nipride, nitroglycerin) or *via* increased substrate required for NO synthesis (i.e. arginine). The delivery of these molecules protects from IR injury in the heart *via* at least two mechanisms [24]. First, NO activates the soluble guanylyl cyclase, which in turn activates a host of cGMP kinases such as protein kinase G, ion channels (e.g. Mito K_{ATP} channel) and phosphodiesterases (PDEs) [51, 52]. The end results are vasodilation and decreased platelet adhesion. Second, NO has direct effects on the binding of neutrophils independent of its influence on cGMP activity [48]. Results from mice wherein endogenous NOS inhibition or the eNOS enzyme has been manipulated underscore the importance of NO bioavailability in contributing to IR injury.

Consequences of Reduced NO Bioavailability : Evidence from Studies of the Endogenous NO Inhibitor Asymmetrical Dimethylarginine (ADMA)

Evidence exists that endogenous NO synthase inhibitors contribute importantly to ischemia and reperfusion injury. Reduced bioavailability of NO can result from reduced NO production and/or increased NO degradation. Impaired NO biosynthesis might result from increased levels of asymmetrical dimethylarginine (ADMA). ADMA is an endogenous competitive inhibitor of NOS [53]. ADMA is elevated and closely correlated with impaired vasodilator function [53]. Bae *et al.* [54] observed increased ADMA concentrations in acute coronary events independent of other cardiovascular risk factors. ADMA is metabolized by the enzyme dimethylarginine-dimethylaminohydrolase (DDAH) [53]. Two isoforms of this enzyme exist; DDAH-1 and DDAH-2. Both isoforms influence NOS function and subsequent endothelial function. Rationale exists for ADMA to influence endothelial function subsequent to IR because systemic administration of other NOS inhibitors either before ischemia or prior to reperfusion increases infarct size, and these effects can be reversed by administering L-arginine [55].

In light of this rationale, Stuhlinger *et al.* [56] determined whether: 1) ADMA is elevated in response to myocardial IR; 2) ADMA contributes to the severity of reperfusion injury; and 3) DDAH activity/expression modulates tissue ADMA levels and the subsequent damage in response to IR. Coronary artery ligation for 30-min followed by 4 h reperfusion in hearts from C57Bl6 mice evoked several well-accepted indices of tissue IR injury. In the same hearts, NO-metabolites were lower and ADMA was higher in tissue obtained at 4 h of reperfusion *vs.* 30-min of ischemia. Further, myocardial DDAH was suppressed at 4 h of reperfusion *vs.* 30-min of ischemia. These data, together with findings that indices of reperfusion injury (e.g. expression of adhesion molecules, leukocyte distribution and activity, and infarct area) in response to the same IR protocol could be blunted: by L-arginine administration; and in DDAH-1 overexpressing mice, provide solid evidence that the endogenous NOS inhibitor ADMA contributes importantly to IR injury.

Studies from the same laboratory provide evidence that ADMA might have clinical relevance with regard to graft

coronary artery disease (GCAD) [57]. GCAD is the leading cause of death after the first year of heart transplantation [58]. The authors tested the novel hypothesis that lowering ADMA concentrations by DDAH overexpression in recipient mice might suppress GCAD in cardiac allografts. Donor hearts were heterotopically transplanted into transgenic mice overexpressing DDAH-1. DDAH-1 recipients had lower ADMA, together with indices of reduced oxidant stress, inflammatory cytokines, adhesion molecules, and chemokines. Importantly, indices of GCAD (e.g. luminal narrowing, intima-media ratio, and percentage of diseased vessels) were markedly reduced in DDAH-1 transgenic *vs*. wild type recipients. The authors concluded that protection from GCAD in DDAH-1 overexpressing mice resulted from increased NO bioavailability subsequent to greater metabolism of ADMA in the transgenic animals [57]. Taken together, strategies to reduce levels of ADMA might be therapeutic in GCAD [57] and IR injury [56].

Consequences of Reduced NO Bioavailability: Evidence from eNOS Null Mice

Cooke *et al.* provide solid evidence that eNOS is protective regarding myocardial injury subsequent to 30-min ischema followed by 4 h reperfusion. As such, it is not surprising that IR injury reportedly increased in eNOS-/- mice with deletion of the NADPH ribose and adenine binding sites (i.e. "Harvard eNOS-/- mice" [59]) *vs*. their WT littermates in response to a similar protocol [60]. However, conflicting evidence exists. For example, Guo *et al.* reported similar infarct size between Harvard eNOS-/- mice and their WT littermates and mice with deletion of the calmodulin-binding domain [i.e. "University of North Carolina (UNC) eNOS-/- mice"] [61] and their WT littermates [62, 63]. Interestingly, protection from IR injury was reported to occur *in vivo* [60] and *in vitro* [64] possibly *via* increased iNOS expression in UNC eNOS-/- mice. Therefore, the contribution from eNOS to IR injury in naïve hearts i.e. no ischemic preconditioning (PC) is equivocal.

PC of the heart refers to the protection afforded by multiple brief ischemic episodes prior to a sustained ischemic insult" [65, 66]. The protective effects of ischemic PC occur in two distinct phases: an early phase that develops rapidly after the stimulus but dissipates within 2-3 h, and a late phase that becomes apparent 12-24 h later and persists for approximately 72 h [67-79]. While there appears to be consensus that the *late* phase of ischemic preconditioning is regulated by eNOS-derived NO [62, 74, 80], the role of eNOS in *early* PC is uncertain. For example, studies using pharmacologic inhibition of PI3K, Akt, and / or eNOS have shown evidence that eNOS activation is [81] or is not [82-84] an important contributor to the early phase of PC. Recently the PC effect of one or six cycles of 4-min occlusion / 10-min reperfusion prior to 30-min occlusion / 4 h reperfusion was evaluated in UNC eNOS-/- mice. Because both PC protocols were equally effective at lessening infarct size in UNC eNOS -/- and WT mice, the authors concluded that basal eNOS activity is not necessary for the early phase of ischemic preconditioning in the mouse. Collectively, while the contribution from basal eNOS activity to modulating infarct size in response to IR injury in naïve hearts is equivocal, it appears that basal eNOS activity is not necessary for

the cardioprotective effects of the early phase of ischemic PC. This latter conclusion is, however, controversial and warrants further investigation.

Consequences of Ca^{2+} Imbalance

Ca^{2+} homeostasis is important in modulating endothelial cell IR injury. Under physiological conditions, Ca^{2+} is maintained in a narrow range in both the cytosol and mitochondria [7, 85]. IR injury is associated with intracellular Ca^{2+} overload in ECs and CMs, although the mechanisms might be different depending on the cell type [85]. During ischemia, intracellular Ca^{2+} increases slowly and during reperfusion the Ca^{2+} increases further. The contribution from Ca^{2+} overload to IR–induced myocardial dysfunction and coronary vascular dysfunction has been assessed in pigs and rats, respectively[86-89] using the NHE subtype 1 isoform (NHE1) inhibitor 4-isopropyl-3-methylsulfonyl-benzoyl-guanidine methanesulfonate HOE642 (cariporide, Aventis Pharmaceutical; Frankfurt, Germany]. Cariporide is an inhibitor of the Na$^+$-H$^+$ pump that in turn decreases harmful intracellular Ca^{2+} overload. In isolated porcine hearts cariporide preserved cardiac function relative to untreated hearts subjected to IR injury. Likewise, IR-induced reductions in systolic wall thickening (sonomicrometry) were delayed and less severe in conscious pigs that were pre-treated with cariporide *vs*. vehicle [88]. Ca^{2+} homeostasis may also be involved in vascular dysfunction as pretreatment with cariporide preserves EC function in coronary arteries upon exposure to IR. Specifically, coronary arteries (approx 115 μm, internal diameter) were obtained from the risk area of rat hearts previously exposed to IR and vascular function was assessed using isometric myography (Fig. **1**) [89]. Coronary vasorelaxation to acetylcholine but not sodium nitroprusside was depressed after IR injury, suggesting an IR-induced vascular defect existed that was specific to the endothelium. Specifically, in vessels from rats pre-treated with cariporide and exposed to the same IR protocol, acetylcholine induced vasorelaxation was impaired to a lesser degree [89]. Isolated vessel preparations (e.g. isometric or isobaric myography) are useful to evaluate mechanisms that contribute to endothelial and/or vascular smooth muscle dysfunction because they are independent from neural, humoral, hormonal, and mechanical influences. However, the situation *in vivo* is not devoid of these influences. Therefore, extrapolation of results using *in vitro* preparations of arteries obtained from experimental animals to *in vivo* pathophysiological conditions in humans should be done cautiously. Nevertheless, results from experiments using rats and pigs suggest that NHE1 inhibitors have important therapeutic potential in settings where IR is likely to occur. Furthermore, the benefit likely is conferred upon both CMs and ECs [88, 89].

ENDOTHELIAL AND MYOCARDIAL INJURY

ECs are uniquely situated to modulate cardiomyocyte function. ECs comprise the inner layer of arteries, veins as well as the endocardial epithelium, and myocardial capillaries. The latter two are critical in regulating cardiomyocyte function [3, 4]. Although CMs are large cells and represent about 75% of intracellular cardiac volume, they comprise a minority of the total cell number. In a normal heart, a capil-

lary is next to almost every CM, and ECs outnumber CMs by approximately 3:1 [3, 4]. Although only a monolayer, ECs, especially in the endocardium, demonstrate a high degree of overlap and interdigitation at cell-cell junctions. Additionally, in the left ventricle of the adult rat, both the intercapillary distance (~15 -20 μm) and the distance between ECs and myocytes are short (~ 1-2 μm) [3, 4]. Rather than being just a passive pathway for delivery of blood, nutrients and oxygen to myocytes, ECs have important and unique regulatory functions on myocardial function and can initiate a host response. Each of these will be elaborated upon.
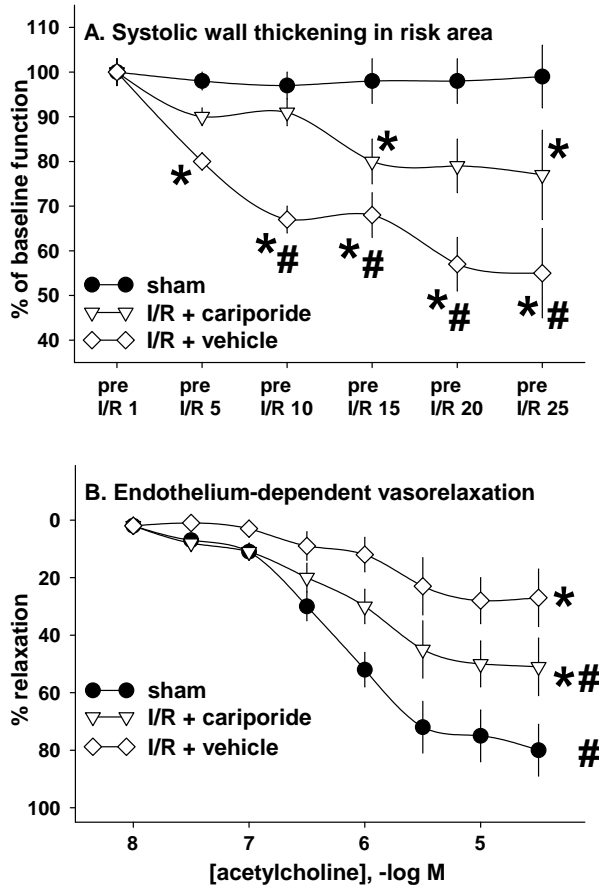

Fig. (1). Panel A. Pretreatment with the sodium-hydrogen exchanger isoform 1 (NHE1) using cariporide attenuates regional dysfunction in response to 25 cycles of ischemia (I, 2-min) and reperfusion (R, 8-min) of the left circumflex coronary artery (LCx) in conscious swine. Reversible occlusions of the left circumflex coronary artery were performed using a hydraulic occluder. Systolic wall thickening (sonomicrometry) was assessed in the normally perfused (left anterior descending, LAD) and risk (left circumflex coronary artery, LCx) regions. Six animals completed three protocols each separated by one week: 1) I/R + VEHICLE (25 I/R cycles + saline infusion); 2) I/R + CARIPORIDE (25 I/R cycles + Cariporide); and 3) SHAM (vehicle administration for 4.2 hours i.e. the occluder was never inflated). Systemic hemodynamics were similar among and within each protocol (data not shown). Blood flow measured during LCx occlusion (microspheres) confirmed that perfusion was reduced (p < 0.05) in the LCx *vs.* LAD regions. During I/R + VEHICLE, LCx systolic wall thickening was reduced (*p

< 0.05) after five IR cycles, and a stable reduction (approximately 55% of baseline; p < 0.05) was present after 20 I/R cycles. During I/R + CARIPORIDE, LCx systolic wall thickening was reduced (*p < 0.05) only after 15 and 25 I/R cycles (approximately 80 and 72%, respectively). The decrease in systolic wall thickening was greater in I/R + VEHICLE *vs.* I/R + CARIPORIDE (#p < 0.05). LCx systolic wall thickening was not altered during SHAM, while LAD systolic wall thickening was similar within and among all protocols. *These findings indicate NHE1 inhibition delays the onset and limits the degree of regional dysfunction in response to repeated bouts of ischemia and reperfusion.* **Panel B.** Pretreatment with cariporide limits I/R –induced coronary microvascular stunning. Anesthetized rats (~ 10 per group) completed 2 × 10-min coronary artery occlusions separated by 5-min of reperfusion, followed by 15 or 60 min of reperfusion (I/R). Cariporide or vehicle was administered 15 min before ischemia (or sham-operation) and was continued throughout each protocol. After reperfusion, hearts were excised, and the reactivity of resistance arteries (internal diameter, ~115 μm) was assessed. The maximal response to acetylcholine-induced relaxation was blunted (*p< 0.05) in I/R + VEHICLE (~35%) and I/R + CARIPORIDE (~55%), compared with sham-operated animals (~85%). However, the percent relaxation to acetylcholine was greater (#p<0.05) in vessels from I/R + CARIPORIDE and SHAM-operated rats *vs.* I/R + VEHICLE animals. Relaxation to sodium nitroprusside was not different among groups (data not shown). Results were similar in vessels obtained from animals after 60 min or 15-min of reperfusion. *These findings indicate NHE1 inhibition before coronary occlusion lessens ischemia-induced microvascular dysfunction for 15-60 min after reperfusion.* This figure is reprinted with permission from the American Physiological Society (APS) from "Na+/H+ exchange subtype 1 inhibition reduces endothelial dysfunction in vessels from stunned myocardium" Am J Physiol Heart Circ Physiol 2001 281: H1575-H1582, APS identifier H925-0].

The Endothelium has Important Regulatory Functions on the Myocardium

ECs modulate CM growth, metabolism, contractility, apoptosis and arrythmogenicity by synthesizing and releasing a variety of signaling molecules. In addition to its vasodilatory effects, NO functions as a signaling molecule. Although NO has a very short half life, because of the close proximity between ECs and CMs, NO is able to diffuse to CMs and regulate contractility, electrical activity, and oxygen consumption of CMs [3]. Other EC signaling molecules include factors responsible for vasoregulation (e.g. prostacyclin, endothelin-1, and angiotensin II,), inflammation (e.g. IL-1, TNF-alpha), and growth [e.g. vascular endothelial growth factor (VEGF), and platelet derived growth factor (PDGF)]. Major signaling molecules associated with ECs are outlined in Table **2**.

EC Injury Correlates with Myocardial Injury

We and others have demonstrated in an isolated heart model that immediately following an IR insult, vascular function strongly correlates with cardiac function [86, 98, 99]. In isolated rat hearts pretreated with verapamil prior to subjecting the organs to 30 minutes of no flow, preservation of coronary flow was significantly correlated with functional recovery (Fig. **2**). These improvements were associated with

decreases in histopathological markers (NF-kB translocation and apoptosis) in EC [98]. In both treated and untreated hearts, minimal cardiomyocyte damage was noted. Although the actual mechanism(s) whereby preserved endothelial function leads to cardioprotection is unclear, hypotheses include enhanced microcirculation, decreased capillary permeability and interstitial edema [99]. In summary, IR injury is associated with decreased vasodilation, which in turn correlates with decreased myocardial function.

Table 2.   Signaling Molecules Associated with Endothelial Cells and their Effects

Modulator	Effect Related to IR
NO	Myocardial contractility (Negative inotrope) [24, 90] Arrhythmias [91] Decreased Myocardial Oxygen Consumption [92]
Prostacyclins	Prevent Oxidative Damage [93]
Endothelin-1	Cardiomyocyte hypertrophy/apoptosis [94]
IL-1 and TNF-alpha	Chemo attractant for neutrophils [95]
VEGF	Prevent cardiomyocyte apoptosis [96] Decreases endothelial permeability [97]
PDGF	Prevent cardiomyocyte apoptosis [95, 98]

Injured Endothelium Initiates Host Response

Neutrophils, platelets and plasma proteins modulate the extent of myocardial injury and inflammation after an IR event. ECs are critical in activating these blood elements. Normally ECs form a barrier against inflammation by preventing neutrophil and platelet activation. Injury upregulates the expression of multiple surface proteins on ECs that lead to binding of leukocytes, platelets, and plasma proteins to the EC surface; this in turn leads to inflammation and further damage. The role of neutrophils, platelets and plasma proteins in IR injury has been detailed elsewhere [10, 100]. This article focuses on the role of ECs in activation of these various effectors.

The EC surface proteins that are associated with IR injury are known as selectins and immunoglobulin cell adhesion molecules (CAM), which have different time courses of activation and expression (Table **3**). The earliest adhesion molecule expressed is P-selectin, which is constitutively expressed and stored in Weibel-Palade bodies located in the cytosol, and translocated to the EC surface within seconds or minutes of reperfusion [24]. P-selectin is responsible for initial leukocyte binding on the endothelial surface. In an *in vivo* feline infarct model, the infusion of anti-P-selectin antibody prior to reperfusion reduces infarct size by approximately 50% [101]. Additionally, *in vitro*, anti-P-selectin antibody treatment preserved endothelial response to acetylcholine, $NaNO_2$, and other vasodilators [102]. Lastly, anti-P-selectin antibody treatment was shown to decrease neutrophil binding to ECs *ex vivo*. In a recent study, delivery of anti-P-selectin antibody *via* the coronary sinus resulted in a significant reduction in infarct size [103]. ICAM-1, a member of

the CAM superfamily is expressed on ECs four to six hours after reperfusion. Both P-selectin and ICAM-1 are associated with leukocyte activation, but ICAM-1 may be more important than P-selectin in initiating the migration of leukocytes across the endothelium and into the myocardium. As with the anti-P-selectin antibody treatment, anti-ICAM-antibody treatment prior to the onset of reperfusion reduced infarct size, preserved vasodilatory response in isolated coronary rings, and decreased binding of leukocytes in a feline infarct model [104]. Similar beneficial effects have also been found in a rat infarct model [103]. However, at present no supporting data are available from human clinical trials for either anti-P-selectin or anti-ICAM-1 antibodies in treatment of myocardial ischemia and reperfusion.
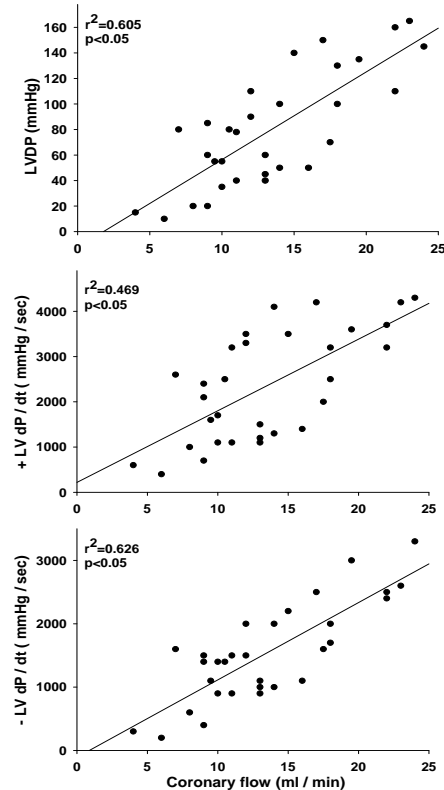

Fig. (2). Correlation between cardiac function and coronary flow. Isolated hearts were perfused at 80 mmHg for 30-min with Krebs-Henseleit buffer at 37 °C. Next, hearts completed 30-min of no-flow ischemia followed by 60-min reperfusion. Cardiac functional measurements were obtained immediately at the end of reperfusion. Left ventricular (LV) developed pressure (LVDP, mmHg), and positive and negative (-) LV dP/dt (mm Hg/sec) correlated significantly with coronary flow [98].

Although less well studied, ECs are important activators of platelets and plasma proteins. Similar to leukocytes, platelets bind to a variety of EC surface receptors including P- and E-selectin, glycoproteins, and the fibronectin receptor. Platelet activation in turn can lead to further upregulation of both selectins and ICAM through the release of pro-inflammatory proteins from platelet granules. Normally ECs are the only natural anti-thrombogenic barrier. Platelets are strongly attracted to and activated by the underlying subendothelial structural molecules including collagen and Von Willebrand factor. Similarly, activated complement complex,

C5b-C9 – the membrane attack complex, is found early after reperfusion on the EC surface [105]. The rapid activation of complement in injured human umbilical vein endothelial cells (HUVECs) appears to occur through the activation of the classical pathway by activation of C3 and possibly by direct activation of C5 due to increased free radicals [106]. CD46 and CD55 are EC surface receptors that are protective from C3 activation, and they appear to decrease after IR injury *in vitro* [107]. In turn, both platelets and complement appear to be important in further activating leukocytes. In summary, strong evidence exists that EC surface proteins activate leukocytes, platelets and complement, which in turn might contribute importantly to the severity of IR injury.

Table 3.   **Endothelial Cell Surface Receptors and Activation of Leukocytes, Platelets, and Complement**

Blood Element	Endothelial Cell Surface Receptors/Activator
Leukocytes	P-selectin E-selectin ICAM PECAM
Platelets	P-selectin E-selectin Vibronectin receptor Glycoproteins
Complement	CD46, CD55, CD59 (protective)

POTENTIAL THERAPEUTIC APPROACHES

Recently, the reperfusion injury signaling kinase (RISK) pathway, has been ascribed as a putative common pathway whose activation may be protective from IR injury [14, 108]. The RISK pathway involves multiple parallel signaling pathways and is a good framework for understanding the pathophysiology of IR induced cardiac injury and developing novel therapeutic approaches (See Fig. **3**). The RISK pathway has been extensively studied in both CMs and hepatocytes, but we believe that the findings might be just as relevant in ECs [14, 109, 110]. The central components of the pathway are phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt) and p42/p44 extra-cellular signal-regulated kinases (Erk 1/2) [14, 108]. Phosphorylation of PI3K–Akt activates multiple downstream substrates: glycogen synthase kinase-3, apoptotic proteins (BAD, BAX, p53 and caspases), eNOS and PKC. Akt kinase specifically appears to be critical in IR induced injury. Erk 1/2 are among a family of serine–threonine kinases that control cell proliferation and survival. The final and irreversible step in IR-induced cell death is the mitochondrial permeability transition pore (MPTP) opening [108, 111]. Below we will further expand on this pathway and the commonality of three potential approaches to the prevention of IR injury: (1) pharmacological (pre)treatment, (2) preconditioning and (3) postconditioning in terms of activating the RISK pathway.

RISK Pathway in EC

The RISK pathway is critical in reducing whole heart and EC IR injury. Support for this statement is provided by two studies. First, isolated rabbit hearts were subjected to 30-min of regional ischemia and 2 hours of reperfusion with and without pretreatment with insulin, a global activator of PI3K [112]. Infarct size was determined by triphenyltetrazolium staining and expressed as a percentage of the area at risk. Second, isolated perfused mouse hearts completed 35-min of global ischemia and were reperfused for 30 min with and without pretreatment with atorvastatin, an EC specific activator of PI3K [113, 114]. Despite difficulties in comparing results from experiments using different species, the similarity of protection afforded by atorvastatin and insulin is quite remarkable (see Table **4**).

Table 4.   **Effect of Treatment (Atorvastatin or Insulin) ± Wortmannin on Infarct Size (Expressed as a Percent of Area at Risk) in Isolated Heart Preparations**

Treatment	Infarct Size		
	Control	Treatment	Treatment + Wortmannin
Statin (mouse)	33±2	16±2	31±2
Insulin (rabbit)	32±2.3	14.7±2.1	28.3±2.2

Postconditioning also is protective from IR injury *via* PI3K-Akt activation. Postconditioning induces shear stress in EC which can precipitate Akt activation [90, 115-117]. In a series of preconditioning experiments in human brain microvascular endothelial cells, activation of PI3-kinase/Akt was necessary to protect endothelium from apoptotic cell death [116]. Causal evidence for the RISK pathway in preconditioning was demonstrated by the PI3-kinase inhibitor LY-294002. PI3K inhibition prevented Akt phosphorylation and reversed the protective effects of preconditioning. Similarly, the red wine antioxidant Resveratrol, a known activator of Akt, in part generates its protective effects on the endothelium [118]. In summary, the RISK pathway is a common pathway that is protective not only in cardiomyocytes but also in ECs of which further detail will be provided below.

Insulin, RISK Pathway and Myocardial Injury

Insulin regulates cardiac metabolism and is an upstream regulator of the RISK pathway. As such, insulin represents an important pharmacological approach and is an excellent tool for understanding the RISK pathway. For example, when insulin receptors are absent in CMs glucose oxidation and fatty acid oxidation are altered such that cardiac function is attenuated [119, 120]. When insulin binds to its receptor a complex signal transduction network is activated that regulates numerous cellular functions [121, 122]. The activated insulin receptor is trans-autophosphorylated on multiple tyrosine residues in its cytosolic domain. The activated insulin receptor recruits and phosphorylates insulin receptor substrate (IRS) proteins. IRS phosphorylation leads in turn to its binding to the p85 regulatory subunit of PI3K [123] which then activates the catalytic subunit p110 to generate phosphoinositol 3,4,5 triphosphate (PIP3). The latter recruits 3-phosphoinositide-dependent protein kinase-1 (PDK-1), which results in its activation. Activated PDK-1 phosphorylates and activates downstream targets such as Akt and phosphokinase C (PKC). In addition to PI3K-dependent insulin

signaling, another downstream limb involves IRS-1 activation of small GTPase Ras, which then activates Raf kinase, the MAP kinase (MKK1) and ERK 1/2. PI3K, Akt and ERK1/2 are part of the RISK pathway.

Insulin receptors are expressed in CMs and in a variety of other cells in the heart including ECs. Indeed insulin receptors are expressed in ECs of both large and small blood vessels [124, 125]. In ECs, the PI3K/AKT pathway mediates an anti-apoptotic effect and also results in an increase in gene expression and activation of eNOS [120, 125-127]. Insulin has been suggested to act on the endothelium to produce potential mediators of hormone action, such as NO and cGMP [125, 128, 129]. Knockouts of the insulin receptors in ECs have altered ET-1 and eNOS expression.

No flow ischemia was shown to inhibit insulin signaling *via* the reduction of intracellular pH [130]. Insulin and insulin like growth factors (IGF) protect hearts from IR injury [131, 132]. These effects are mediated *via* activation of PI3K. In parallel, Akt activation has been shown to prevent injury after transient cardiac ischemia *in vivo* [133]. In contrast, chronic activation of Akt was deleterious to the heart during the onset of ischemia in an ex-vivo mouse model. In this study, over-expression of an activated form of Akt prevented hearts from recovery after ischemia. However, restoration of PI3K reduced ischemic injury in Akt transgenic hearts [134]. It is unclear how activation of insulin signaling, whether prior to ischemia or at reperfusion, limits damage caused by ischemia. It has been proposed that, the activation of Akt is pro-survival and can limit cell death [131, 133]. The latter study also proposed that activation of Akt increases glucose transport after ischemic injury to the same level seen with insulin stimulation. Further, insulin was shown to phosphorylate a proapoptotic molecule which in turn limits cell death [132].

An extensive work has been conducted to understand the downstream targets of insulin-mediated cardioprotection. Baines and coworkers have shown that insulin mimics the cardioprotection afforded by ischemic PC (discussed above and below) and that both insulin treatment and ischemic PC involve PI3K activation [112]. However, insulin-mediated cardioprotection is independent of activation of both PKC and mitochondrial ATP-sensitive channel [112]. The cascade initiated by ischemic PC-activation of PI3K required PDK1, because hearts from PDK1 hypomorphic mutant mice could not be preconditioned. The signaling pathway involving PDK1 converges to AKT and glycogen synthase kinase 3β (GSK3β) activation and a consequent change in mitochondrial membrane potential [135]. There is increasing evidence that mitochondria-mediated death pathway occurs in the heart as a result of IR injury [136-138]. The MPTP plays a central role in this process [139]. Insulin signaling has been postulated to modulate the MPTP opening. Indeed, insulin-mediated GSK3β inhibition is involved. The exact mechanism by which inhibition of GSK3β modulates insulin mediated-MPTP function is still unknown.

Role of MPTP in Endothelial Cell Injury

As noted the final step is MPTP opening. The MPTP consists of three proteins, adenine nucleotide translocator (ANT), voltage dependent anion conductance (VDAC) and cyclophilin D (CyD) that span the inner and outer mitochondrial membranes and have a pore size of 1500 Da (Fig. **4**). ANT spans the inner membrane and is primarily responsive to the ligands ADP, ATP and dADP, and translocates ADP into and ATP out of the mitochondria. VDAC spans the outer mitochondrial membrane and appears to have a role in transporting metabolites. CyD appears to have an important regulatory role in pore opening and confers cyclosporine (CSA) sensitivity. During steady state conditions the MPTP is closed and allows the maintenance of a mitochondrial membrane potential that generates ATP *via* oxidative phosphorylation. CSA is an important tool in understanding the role of MPTP IR injury. For example when CSA binds to CyD MPTP pore opening is prevented and IR injury is minimized [137, 140]. Mice lacking CyD are more resistant than wild type mice to IR injury [141, 142]. MPTP appears to be critical in cell death from Ca^{2+}, oxidative stress and ischemia but not radiation [141].

The MPTP is a nonspecific pore whose opening is associated with cell death whether necrotic or apoptotic [12, 32, 109, 136, 143-145]. Initiation of MPTP opening may occur through an "extrinsic" or ligand mediated activation of a death receptor or through an "intrinsic" pathway. The "extrinsic" pathway is mediated by tumor necrosis factor-α or FAS to cell surface receptors [12]. The intrinsic pathway is mediated by imbalances in cellular metabolism such as increased ROS, acidosis, and increased intracellular Ca^{2+} all of which have been discussed above. The severity of damage is dependent on the duration of MPTP opening and the number of mitochondria involved. For example, the MPTP may: 1) open briefly or flicker resulting in no loss of mitochondrial membrane potential; 2) open longer but then close leading to apoptosis via release of apoptotic factors discussed below; or 3) open and remain open resulting in loss of mitochondrial membrane potential and eventually necrosis. Overall, the severity of damage is dependent on the duration of MPTP opening and the number of mitochondria involved. Major consequences of MPTP opening are loss of mitochondrial membrane potential and release of apoptotic factors (Table **5**). Of the apoptotic factors released as a consequence of MPTP opening, Cytochrome C may be the most important leading to activation of Caspase 9, and a consequent destruction cascade beginning with activation of Caspase 3. In addition to release of proapoptotic factors and loss of the mitochondrial membrane potential, other deleterious consequences of MPTP opening include depletion of ATP, elevation of mitochondrial Ca^{2+}, and increased mitochondrial ROS formation. The end result is cell death and loss of oxidative phosphorylation.

Pharmacological Pretreatment

Drug treatment is often protective when administered prior to ischemia [86, 88, 89, 98, 99, 111, 147-154]. These drugs include insulin, adenosine and adenosine agonists, bradykinin, statins, cariporide, cyclosporine (CSA), and sanglifehrin A (SFA). All except for CSA and SFA likely work upstream from MPTP opening. In contrast, CSA and SFA directly regulate the MPTP. None of these, except for insulin, has been tested clinically [152, 153]. In summary, *in vitro* and *in vivo* models demonstrate that pharmacological treatment can be protective from IR injury.
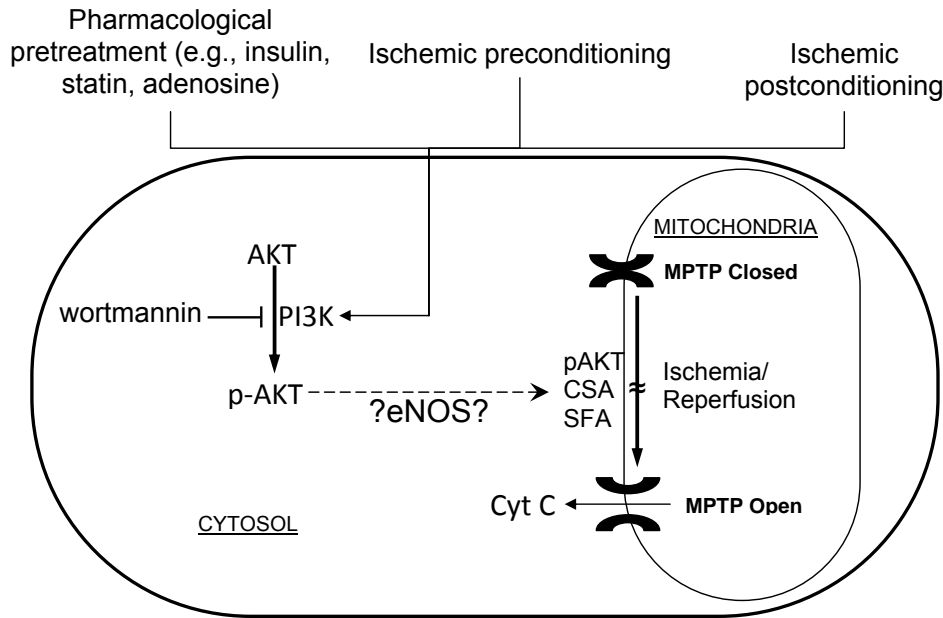
Fig. (3). The reperfusion injury signaling kinase (RISK) pathway. Pharmacological agents, ischemic preconditioning and/or ischemic post-conditioning can activate PI3K to precipitate Akt phosphorylation. Akt phosphorylation might prevent opening of the mitochondrial permeability transition pore (MPTP) *via* endothelial nitric oxide synthase (eNOS). This would be protective because MPTP opening is believed to be an irreversible step toward cell death. CSA, cyclosporine; SFA, sanglifehrin A.

Ischemic PC

As mentioned earlier, ischemic PC was first described in 1986 as "multiple brief ischemic episodes (that) might actually protect the heart from a subsequent sustained ischemic insult" [65, 66]. In the landmark paper, myocardial ischemia evoked by occlusion of the circumflex coronary artery for either 40-min or three hours after no treatment (control) or four prior bouts of 5 minutes of ischemia alternating with 5 minutes of reflow (pretreatment). Infarct size evoked by 40-min of ischemia was reduced from 30% to 7% of the area at risk in control *vs.* pretreated dogs, respectively; however, infarct size evoked by three hours of ischemia was similar between groups. In 2000, Tong and coworkers demonstrated that inhibition of PI3K, an important component of the RISK pathway, negated the protective effects of preconditioning [155]. Briefly, Langendorff-perfused rat hearts were perfused for 20 minutes (control) or treated with 4 cycles of 5-minute ischemia and 5-minute reflow (preconditioning) and then treated with either wortmannin or LY 294002 (PI3K inhibitors) for 5 min before and throughout PC. The benefits of ischemic PC on left ventricular developed pressure after 30-min of reperfusion were abolished by PI3K inhibition. These data, combined with results showing that ischemic PC resulted in Akt phosphorylation of whole heart homogenates underscores the importance of the PI3K pathway in preconditioning. The importance of MPTP was demonstrated similarly in isolated rat heart subjected to preconditioning [9, 13]. The beneficial effects of preconditioning could be negated by pretreatment with atractyloside, an MPTP opener. In neonatal rat CMs, the direct relationship between Akt and MPTP has been demonstrated using a combination of western blotting and confocal microscopy.

Table 5. Putative Apoptotic Factors Released at MPTP Opening

Effectors of MPTP Opening	Consequence
Cytochrome C release	Activation of Caspase 9 which in turn activates Caspase 3 [144, 146]
Apoptosis Inducing Factor (AIF)	Translocates to nucleus and activates DNAses [146]
Second mitochondria-derived activator of Caspase/direct inhibitor of apoptosis binding protein with a low PI (smac/DIABLO)	Inhibits inhibitors of apoptosis – in the end proapoptotic [146]
Heat Shock Proteins	Loss of ability to generate ATP [146]
Ca^{2+} release	Loss of ability to generate ATP [146]
ROS generation	Amplification of MPTP opening [32]

Although the above studies demonstrate that ischemic PC is effective in whole heart preparations, endothelial dysfunction has been specifically demonstrated in experimental models of IR injury [9]. Kharbanda and coworkers examined the effects of forearm ischemia on radial artery flow and demonstrated vasoconstriction in the radial artery after 20 minutes of ischemia, which could be prevented by preconditioning. In addition, the authors reported less neutrophil acti-

vation in preconditioned human subjects, which they linked to decreased endothelial injury. In another model of human forearm ischemia, infusion of L-arginine (substrate for NO) was protective in response to ischemia [156]. Taken together, these data suggest that preconditioning has a specific endothelial benefit.
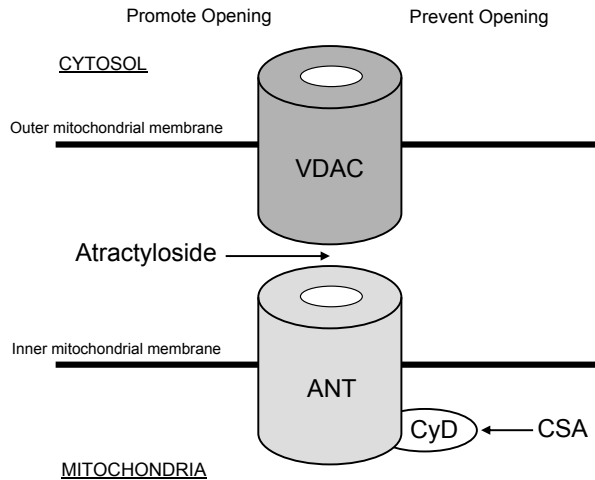

Fig. (4). The mitochondrial permeability transition pore (MPTP) consists of three proteins: adenine nucleotide translocator (ANT); voltage dependent anion conductance (VDAC); and cyclophilin D (CyD). When cyclosporine (CSA) binds to CyD pore opening is prevented and IR injury is limited. Atractyloside is an MPTP opener that exacerbates IR injury.

Postconditioning

Postconditioning is defined as repetitive bursts of ischemia applied during early reperfusion, which attenuate myocardial reperfusion injury. Postconditioning was originally described in dogs where the left anterior descending artery (LAD) was occluded for 60 minutes and reperfused for three hours [157]. In control dogs there was no intervention, in preconditioned dogs the LAD was occluded for 5 min and reperfused for 10 min before the prolonged occlusion, and in postconditioned dogs, at the start of reperfusion, three cycles of 30-s reperfusion and 30-s LAD reocclusion preceded the 3 h of reperfusion. Both preconditioning (15%, P =0.05) and postconditioning (14%, P=0.05) decreased infarct size compared to controls (25%). In addition, neutrophil accumulation and basal endothelial function- assessed by the maximal vasodilator response of post-ischemic LAD to acetylcholine - were improved similarly in vessels from preconditioned and postconditioned dogs *vs.* controls. These observations demonstrate the critical role of the endothelium in preconditioning and postconditioning. Subsequent studies provide evidence for PI3K-Akt activation as a necessary step for the effects of postconditioning to be realized. For example, PI3K inhibitors abolished the benefits of postconditioning [158, 159]. In rabbits, Argaud and coworkers demonstrated that postconditioning, preconditioning and NIM811, a specific inhibitor of the MPTP, reduced MPTP opening and limited infarct size [160]. This study did not specifically detail the effects on endothelium. As with preconditioning, the benefits of postconditioning have been demonstrated in a human

forearm model of ischemia by looking at radial artery function in ischemic and postconditioned subjects [161]. Postconditioned subjects had superior preservation of flow relative to untreated subjects. Postconditioning may also work through the induced shear stress in EC, which is known to activate Akt in EC [90, 115]. Although a direct link between flow and MPTP is not yet established, PI3K-Akt (*via* the RISK pathway) is mechanosensitive and may be another possible mechanism by which postconditioning is protective. Even though the mechanism is not clearly defined, the endothelium plays a critical role in the protective response induced by postconditioning.

CONCLUSIONS

Endothelial cells are uniquely situated to modulate IR injury in the heart. They are able to tolerate long periods of ischemia and upon reperfusion activate a variety of blood elements including neutrophils, platelets and complement. Because of their proximity to cardiomyocytes, they can modulate cardiomyocyte function and injury. Further focus on the RISK pathway in EC may present a deeper understanding of IR injury and provide new opportunities for its treatment or prevention.

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