

Mechanisms of the Beneficial Actions of Ischemic Preconditioning on Subcellular Remodeling in Ischemic-Reperfused Heart

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Abstract: Cardiac function is compromised by oxidative stress which occurs upon exposing the heart to ischemia reperfusion (I/R) for a prolonged period. The reactive oxygen species (ROS) that are generated during I/R incur extensive damage to the myocardium and result in subcellular organelle remodeling. The cardiac nucleus, glycocalyx, myofilaments, sarcoplasmic reticulum, sarcolemma, and mitochondria are affected by ROS during I/R injury. On the other hand, brief periods of ischemia followed by reperfusion, or ischemic preconditioning (IPC), have been shown to be cardioprotective against oxidative stress by attenuating the cellular damage and alterations of subcellular organelles caused by subsequent I/R injury. Endogenous defense mechanisms, such as antioxidant enzymes and heat shock proteins, are activated by IPC and thus prevent damage caused by oxidative stress. Although these cardioprotective effects of IPC against I/R injury are considered to be a consequence of changes in the redox state of cardiomyocytes, IPC is considered to promote the production of NO which may protect subcellular organelles from the deleterious actions of oxidative stress. The article is intended to focus on the I/R-induced oxidative damage to subcellular organelles and to highlight the cardioprotective effects of IPC. In addition, the actions of various endogenous cardioprotective interventions are discussed to illustrate that changes in the redox state due to IPC are cardioprotective against I/R injury to the heart.

Keywords: Cardioprotection, ischemia-reperfusion injury, ischemic preconditioning, oxidative stress, reactive oxygen species, subcellular organelles.

INTRODUCTION

Oxidative stress plays a role in a number of cardiovascular diseases including hypertension, cardiomyopathy, cardiac hypertrophy, heart failure, and ischemia-reperfusion injury (I/R) [1, 2]. The I/R phenomenon pertains to cell damage induced by reactive oxygen species (ROS) and is associated with the development of intracellular Ca^{2+} overload as well as deleterious effects on subcellular organelles [1, 2], (see Fig. (1)). The increase in intracellular Ca^{2+} due to I/R injury consequently triggers a variety of chemical reactions that further augment production of ROS [3]. The primary sources of oxidative stress in the heart are mitochondrial cytochromes, as well as xanthine oxidoreductase, NAD(P)H oxidase, and nitric oxide synthase (NOS) [4]. A positive feedback loop concerning "ROS-induced ROS release" has been proposed after observing that ROS associating with mitochondrial depolarization resulted in a further release of ROS from the mitochondria [5]. This release of ROS changes the intracellular environment from its original reducing state to oxidizing milieu signaling causing the eventual expiration of the cell [6]. In particular, during the ischemic phase the level of ROS is relatively low [7], however upon reperfusion of the ischemic heart, bursts of ROS have been observed using electron paramagnetic resonance spectroscopy, spin trap [alpha]-phenyl-*N-tert*-butylnitron, and luminal-enhanced ter-butyl-initiated chemiluminescence [1].

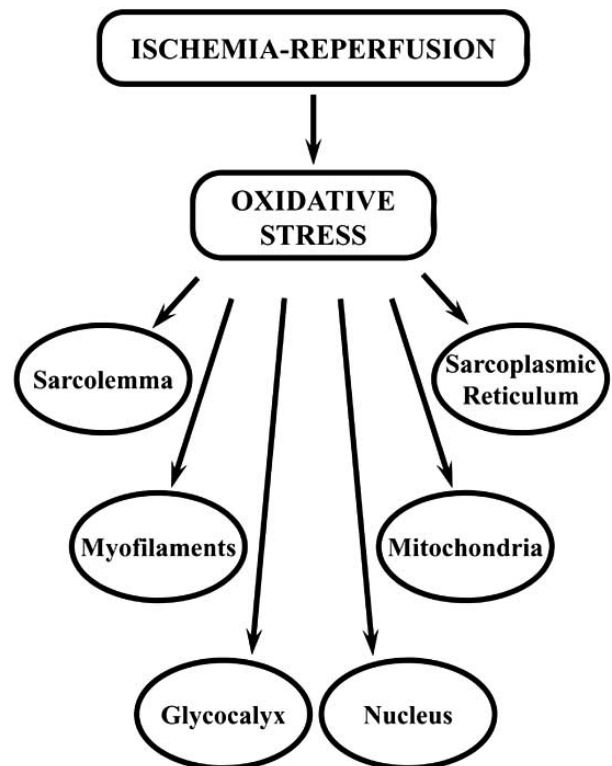


Fig. (1). Subcellular organelles affected by oxidative stress induced by ischemia-reperfusion injury.

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Clinical procedures including angioplasty, thrombolytic therapy, coronary bypass surgery, and cardiac transplantation are considered to cause I/R injury where the ischemic insult is prolonged beyond a certain critical period [1]. It can also occur following the termination of an angina attack [1]. This large burst of ROS following reperfusion can decrease cardiac function by inhibiting contractile activity [8], altering membrane permeability [9], and increasing cell death [10]. The damage caused by I/R injury can potentially be attenuated by cardiac preconditioning, first observed in the canine myocardium [11]. This phenomenon has been extensively studied since its initial detection in 1986 and has been found to be instrumental in delaying myocardial necrosis and reduce cell apoptosis, diminishing I/R-induced ventricular arrhythmias, preserving post-ischemic endothelial function, attenuating neutrophil-mediated inflammation response in the myocardium, and improving contractile function [11, 12]. Ischemic preconditioning (IPC) diminishes the effects of both endogenous and exogenous oxidative stress [13] and limits the cycle of depressive pro-inflammatory cytokine production [14], reducing myocardial infarct size and improving cardiac function. For a summary of possible mechanisms of IPC refer to Table 1. It should be mentioned that IPC is normally produced by subjecting the heart to three to five cycles of brief ischemia followed by brief reperfusion and is now well known to be cardioprotective against I/R-induced injury.

Table 1. Possible Mechanisms for the Beneficial Effects of Ischemic Preconditioning

1.	Low concentrations of ROS and changes in redox state
2.	Activation of PKC signaling
3.	Formation of adenosine
4.	Activation of NOS and production of NO
5.	Activation of sarcolemmal K ⁺ -ATP channels
6.	Activation of mitochondrial K ⁺ -ATP channels
7.	Increase in antioxidant reserve
8.	Activation of heat shock proteins

Abbreviations: ROS, reactive oxygen species; PKC, protein kinase C; NOS, nitric oxide synthase; NO, nitric oxide

ALTERED FUNCTIONS OF SUBCELLULAR ORGANELLES BY ROS AND IPC

During ischemia there is a transition from aerobic to anaerobic metabolism in the heart that leads to a reduction in energy production depleting readily available high energy phosphate stores including ATP and creatine phosphate [15]. As a result, not only is the cardiac contractile function decreased, but also the function of energy-dependent ion pumps which cause an accumulation of metabolites and cations and leads to acidosis, increases the osmotic load, activates Ca²⁺-dependent enzymes and produces ROS [15]. The components of the myocardial cell, which are modified by ROS and ultimately lead to the overall change in myocardial function, include the nucleus, glycocalyx (extracellular matrix), cardiac myofilaments, sarcoplasmic reticulum (SR),

sarcolemma (SL) and mitochondria of the myocardial cell. It is pointed out that excessive amounts of ROS, which are formed during the development of I/R injury, are considered to result in oxidative stress and produce deleterious effects on the myocardium. On the other hand, low concentrations of ROS, which are generated upon subjecting the heart to IPC, result in changing the redox state of cardiomyocytes and produce beneficial actions against I/R injury [12].

Cardiac Nucleus

Modifications of the cardiac nucleus are important in IPC due to the observation that it undergoes changes that contribute to both early phase IPC, occurring 1-3 hours after stimulus, and delayed phase IPC, occurring 12-24 hours after IPC stimulus [16]. Delayed phase IPC is caused by the upregulation of cardioprotective genes that occurs during the initial IPC stimulus which progresses to transcription regulation and then translation [17]. It includes the upregulation of VEGF, for angiogenesis promotion, and the anti-apoptotic Bcl-2 protein family, to attenuate apoptosis. There have also been observed increases in mRNA levels for heat shock proteins (HSP) 27, 70, and 89 as well as antioxidants such as catalase, glutathione peroxidase, and manganese superoxide dismutase (MnSOD) [18]. The transcription factor most extensively studied with regard to I/R injury and IPC is the nuclear factor κ -light-chain enhancer of activated B cells (NF κ B). Elevated mRNA expression and DNA binding of NF κ B has been demonstrated during IPC where, initially its translocation to the nucleus is increased, but then after prolonged I/R its activation is reduced [17, 19]. This has an effect on late-phase IPC by producing nitric oxide (NO) *via* inducible nitric oxide synthase (iNOS) upregulation [12] which leads to cyclooxygenase-2 activation and the production of cytoprotective prostaglandins PGE₂ and PGI₂, as well as MnSOD and the expression of the anti-apoptotic Bcl-2 gene family [20-22]. Observations involving Bcl-2-associated athanogene-1 (BAG-1) illustrate interactions with heat shock proteins HSC70 and HSP70 that may promote cell survival. BAG-1 is normally detected in the nucleus, however following ischemia, not only was the expression of both its isoforms increased, it also increased its binding to HSC70 in rat cardiomyocytes [23]. Its expression was not attenuated following I/R, indicating potential cardioprotective properties. In addition, when undergoing IPC, there has been an observed increase in the expression of the fatty acid transport (FAT) gene as well as genes involved with remodeling (fibronectin, laminin, and collagens I and II) [24, 25]. On the other hand, oxidative stress results in the down-regulation of genes corresponding to energy-generating pathways, such as fatty acid metabolism [25].

Cardiac Glycocalyx & Myofilaments

Oxidative stress and IPC have measurable effects on glycocalyx activities and cardiomyocyte myofilaments. The glycocalyx is particularly susceptible to I/R stress as it is the first to be exposed to injury, although preconditioning has also been partially effective in protecting it [26]. IPC reduces the cleavage of myofilament troponin I by matrix metalloprotease 2 (MMP-2) [27] by decreasing its release and activation. In isolated rat hearts, the activation and release of

MMP-2 has shown direct correlation with cardiac dysfunction observed in I/R injury [27, 28]. Oxidative stress, in particular stress induced by hydroxyl radicals and peroxynitrite, appear to cause damage in myofibrils, previously noted in chronic atrial fibrillation patients [29], although it may also cause a similar effect as a result of I/R injury. The cytoskeleton has also demonstrated responsiveness in IPC *via* ROS activation of p38 MAPK, which subsequently activates HSP 27. Not only does this cause the polymerization of actin filaments, it also increases the stability of the contractile apparatus through late-phase preconditioning [30-32].

Sarcoplasmic Reticulum

The SR is a key regulator of intracellular concentration of Ca^{2+} in cardiomyocytes. It plays an integral role in maintaining cardiac contractile function as a result of its regulation of Ca^{2+} . During I/R injury, it has been observed that cytosolic Ca^{2+} increases dramatically during initial ischemia causing damage to the cell and decreasing ATP [33-35]. Specifically, it has been noted that, even after brief episodes of ischemia, there are changes in SR Ca^{2+} channels [36]. There have also been observed decreases in mRNA levels of SR Ca^{2+} -cycling proteins; however, IPC has been demonstrated to have the capacity to attenuate these changes by preventing intracellular Ca^{2+} overload, therefore upholding normal SR function [37, 38]. The ROS species H_2O_2 has exhibited direct effects on SERCA (sarcoplasmic reticulum Ca^{2+} -stimulated-ATPase) by decreasing its activity and causing NCX (Na^+ - Ca^{2+} exchanger) to increase its activity [39]. This implies that redox-dependent SR Ca^{2+} depletion may be partially affected by this reciprocal regulation of SERCA and NCX in rat ventricular myocytes [39]. Specifically, $\text{FeSO}_4/\text{EDTA}$ -induced oxidative stress has shown modification of $-\text{SH}$ groups, lysine, tryptophan, and tyrosine which are crucial to optimal Ca^{2+} -stimulated-ATPase activity [40]. On the other hand, oxidation of the $-\text{SH}$ groups of SR ryanodine receptors (RyRs) have been implicated as being essential in the IPC process for attenuating subsequent prolonged I/R-induced intracellular Ca^{2+} -overload [41]. IPC induced cardioprotection of the SR involves mediating Ca^{2+} efflux and influx between the SR and the cytoplasm of cardiomyocytes in order to prevent contractile dysfunction. Understanding the differences between oxidative stress induced by I/R injury from the beneficial effects induced by IPC on the SR could be key in deciphering what signaling is required to cause cardioprotection.

Sarcolemma

The phospholipid bilayer boundary of cardiomyocytes, the SL, contains a variety of receptors, as well as cation pumps, exchangers, and channels that are immediately susceptible to redox modification which potentially affects downstream signaling in the cardiomyocyte causing alteration of its normal function. H_2O_2 has been specifically noted to have a bi-phasic effect on ATP-binding where the initial increase and then subsequent decrease of binding of ATP occurs in a time and concentration-dependent manner [42]. In addition, HOCl, a well known oxidant, was shown to inhibit ATP-binding; both these observations were found in SL membranes isolated from rat ventricular cardiomyocytes

[42]. When porcine heart SL was treated with the xanthine-xanthine oxidase, an oxyradical generating system, a decrease in Na^+ - K^+ -ATPase activity occurred which correlated partially with a decrease in its affinity. It was postulated that the inhibitory effect may be partially due to superoxide radical generation as singlet oxygen was found to decrease cardiac sarcolemmal Na^+ - K^+ -ATPase activity [43, 44]. An interesting study by Fuller *et al.* [45] identified an endogenous, stable inhibitor of cardiac-specific Na^+ - K^+ -ATPase that accumulates in the cell during ischemia in rat hearts. It was found that SL Na^+ - K^+ -ATPase activity rose when SL membranes were purified away from the cytosol, where the inhibitor is located. Production of this inhibitor corresponded with oxidative stress induced by ischemia, indicating that oxidative stress has the capacity to inhibit Na^+ - K^+ -ATPase through this mechanism [43, 45, 46]. The activation of Na^+ - K^+ -ATPase increases levels of Na^+ , observed during ischemia, which can be partially explained by the presence of this inhibitor. Interestingly, the inhibitor was unable to be detected in the cardiac effluent post-reperfusion which the authors postulated could be due to it remaining accumulated in the cytosol of the cell, or it may be inactivated immediately post-reperfusion [45]. It has also been observed that oxygen free radicals can physically disrupt the SL membrane, causing the intracellular space to be exposed to the extracellular environment [9]. In addition, oxidative stress, caused by xanthine-xanthine oxidase, altered the activity of SL phospholipase C which ultimately affects downstream signaling, including the activation of PKC [47]. Furthermore, the activity of SL phospholipase D, which primarily is a part of the signal transduction mechanism for regulating Ca^{2+} movements in the heart, was impaired by I/R injury *via* H_2O_2 and HOCl by modification of its functionally critical thiol groups [48]. Also, the thiol groups associated with phosphatidylinositol 4-kinase and phosphatidylinositol 4-P-5 kinase, required for the proper function of numerous SL proteins, were impaired by ROS therefore reducing their function [49].

Despite the previously described ROS-induced alterations of SL proteins, the one most discussed cardioprotective effect of preconditioning is the SL version of K^+ -ATP channels (sarc K^+ -ATP channels) and its comparison with mitochondrial K^+ -ATP channels (mito K^+ -ATP channels). These K^+ -ATP channel proteins are activated by NO either from NO synthase (NOS) or extracellularly available NO [15]. Although the general consensus is that mito K^+ -ATP channels play a more significant role regarding the beneficial effects of IPC, sarc K^+ -ATP channels are important because of an increased vulnerability of these sites to oxidative stress [50]. Sarc K^+ -ATP channels were initially noticed as being involved in IPC as they are opened when exposed to free radicals and have been observed to possess cytoprotective properties [51-54]. In a study investigating the difference between sarc and mito K^+ -ATP channels, sarc K^+ -ATP channels appeared to act as an effector of preconditioning and were found to be important in improving functional recovery. This was indicated when cardioprotective effects induced by sarc K^+ -ATP channels occurred during the stress period, however its activation was not required during the preconditioning period. In freshly isolated adult rat cardiomyocytes, using an isoflurane-induced protection technique

to study the effects of preconditioning [55], both sarc and mito K^+ -ATP channels were observed to be required for cardioprotection against oxidative stress. In a specific mouse model knock-out of Kir 6.2 (the pore subunit of sarc K^+ -ATP channels) cardioprotective effects induced by IPC were extinguished, indicating their necessity for cardioprotection in this model [56]. Finally, it was shown that activated sarc K^+ -ATP channels are important in preventing cardiomyocyte apoptosis and mitochondrial damage during stress, as inhibition of these ATP channels promoted the mitochondrial death pathway by augmenting oxidative stress-induced apoptosis. In addition, it was noted that mitochondrial Ca^{2+} loading was also significantly increased upon inhibition of the sarc K^+ -ATP channel in cultured HL-1 and neonatal cardiomyocytes [57]. Clearly, the oxidative stress-induced alterations on the SL play a significant role in IPC, and despite the discussions comparing the significance between sarc and mito K^+ -ATP channels, it is clear that, not only do the sarc K^+ -ATP channels play an important role in IPC, but other proteins of the SL do as well.

Mitochondria

There is an ample amount of literature discussing how mitochondria are involved in IPC. Mitochondria maintain the balance between cell life and cell apoptosis, where the key to its activity is its generation of ROS and free radicals within the cell. It appears that the majority of pathways currently discussed to be effective in cardioprotection converge on the mitochondrial permeability pore in an effort to keep it closed; specifically to preserve its inner membrane potential and reduce mitochondrial Ca^{2+} to ensure uninterrupted energy production [15]. In the previously discussed study comparing sarc K^+ -ATP and mito K^+ -ATP channels [55], mito K^+ -ATP channels acted as both an effector of IPC and its trigger. Mito K^+ -ATP channels were observed to be activated during the preconditioning period and during the exposure to oxidative stress. In contrast to the sarc K^+ -ATP channels, where they were found to be important for improving functional recovery, mito K^+ -ATP channels were shown to have an effect primarily on infarct size [55]. The pharmacological preconditioning of the rat heart, using diazoxide, has shown to cause the opening of mito K^+ -ATP channels and protect against ischemia-induced ventricular arrhythmias [58]. It was also demonstrated that in the mitochondrial protective pathway, the ROS released from the mitochondria during IPC caused the opening of mito K^+ -ATP channels upstream and was protective [59]. An extensive study carried out by Das and Sarker [60] on mito K^+ -ATP channels by using nicorandil or minoxidil, found that mito K^+ -ATP channels have the potential to protect against I/R-induced arrhythmias, reduce myocardial infarct size, and increase cardiomyocyte survival in the intact anesthetized rabbit heart. The mito K^+ -ATP channels have been observed to have properties that detect local oxidants resulting in decreased free radical generation. These sensors are likely located in the channel's sulfonylurea receptor where stimulation by reactive oxygen prevents mitochondrial ROS release [61]. Although some variations among the results are evident in different animal models, as well as different means of inducing IPC and/or I/R injury, it is clear that mito K^+ -ATP channels play some role in IPC in protecting the heart against I/R injury.

The functions of mitochondria have been shown to be altered upon exposure of the heart to IPC including the expression of the mitochondrial antioxidant, manganese superoxide dismutase (MnSOD), via the formation of NF κ B and activator protein-1 (AP-1) [21]. There is also increased ATP production in preconditioned hearts when compared to non-preconditioned hearts [62]. The phenomenon of "ROS-induced ROS release" involves opening of the mitochondrial permeability transition pore (mPTP) by ROS to release additional ROS, validating the mitochondria as the primary source of ROS in the cell [5, 10]. Interestingly, IPC prevents the mPTP from opening so the burst of ROS does not occur which leads to an overall reduction of oxidative stress in the cell [5]. An intriguing review on the inhibitory effect of IPC on mitochondrial respiratory complexes has discussed how IPC could cause gradual activation of mitochondrial function; ultimately bypassing ROS bursts and Ca^{2+} overload [63]. Complex I (NADH ubiquinone oxidoreductase) represents the electron entry into the mitochondria and is a major site of ROS generation [64, 65]. The regulation of NADH/NAD $^+$ redox balance was shown to influence mPTP opening where an increase in NADH/NAD $^+$ ratio inhibited its opening [63, 66]. IPC and NO also inhibited the activity of Complex I thus minimizing ROS generation [63]. Complex I was reversibly inhibited by S-nitrosation, a potential mechanism for NO-dependent mitochondria respiratory chain control [67]; inhibition of this complex by as little as 25% has demonstrated significant inhibition of the respiratory chain, as it is the entry point for electrons [67, 68]. Complex II (succinate dehydrogenase) has been connected to mito K^+ -ATP channel function where inhibition of complex II was found to open mito K^+ -ATP channels and result in cardioprotection [63, 69]. Complex III (cytochrome bc1 complex) is another site of ROS formation in the electron transport chain, where its inhibition is a function of its own ROS generation [63]. Unfortunately, how this complex is affected by IPC is currently unknown. Complex IV (cytochrome c oxidase) has been shown to be inhibited by NO, which is released during I/R [63, 70]. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also inhibited by endogenously derived nitrolipids generated during IPC [71, 72]. This causes the accumulation of fructose-1,6-bisphosphate (F-1,6-BP) which has been observed to improve glycolytic flux and functional recovery in post-ischemic myocardium [73]. In addition, the quantity of lactate, the final product of anaerobic glycolysis that occurs during ischemia, was observed to be nine-fold less in preconditioned hearts compared to non-preconditioned hearts [73]. This demonstrates the ability of IPC to inhibit glycolysis resulting in the prevention of acidosis [64]. As a final note, cytosolic to mitochondrial relocation of hexokinase has been shown to occur in IPC, moderating cytochrome *c* release and ROS production [74, 75].

ENDOGENOUS CARDIOPROTECTIVE INTERVENTIONS

A number of proteins and molecules are involved in cardioprotection and are influenced by the redox state induced by IPC. The majority of these proteins had, at some point, been involved following the release of small amounts of ROS. Low NO preconditioning of H9c2 (an embryonal rat

heart-derived cell line) cells can induce the production of the cyclooxygenase-2 (COX-2) protein [76]. Although COX-2 is upregulated in oxidative stress-induced injury and apoptosis, it was found to be cardioprotective upon the conversion of arachidonic acid to PGH₂. It is pointed out that PGH₂ is further derived into cytoprotective prostanoids, PGE₂ and PGI₂, which were found to attenuate stunning and reduce infarct size after I/R [77-80]. PKC ϵ has also been attributed to IPC as it is activated by low concentrations of oxygen radicals and has been observed to be involved in IPC cardioprotection [81-84]. The activation of phospholipase (PL) C and D causes the subsequent release of diacylglycerol, a PKC activator, suggesting PKC as a molecule involved in IPC [15]. PLD has been implied to evoke a cell-survival response upon exposure to low concentrations of oxidants for a brief length of time [85]. In particular, PLD 2 has been noted to have elevated activity when reperfusion occurs briefly after a brief period of ischemic insult to the heart, but its activity is reduced during prolonged reperfusion [86]. Preconditioning has also been illustrated as being promoted by various receptors such as adenosine, adrenergic, bradykinin, and opioid receptors as well as limiting the cycling of depressive pro-inflammatory cytokine production [14, 87]. Thioredoxin, a sulfide reductase, plays a part in maintaining the redox activity inside the cell whose activity is propagated by oxidative stress [88, 89]. It has been shown to be instrumental in transmitting the survival signal in ischemic myocardium [90]; however, it is down-regulated after I/R injury [91]. Interestingly, post-IPC appears to cause an upregulation in thioredoxin, and, in transgenic mouse hearts where there are extra copies of its gene, the cardiomyocytes were resistant to apoptosis [91].

Cardiomyocytes have a few antioxidants that are able to protect the cell from oxidative stress. It is important to protect the cell from oxidative stress, both intracellularly and extracellularly, and the first endogenous antioxidant to encounter ROS is extracellular superoxide dismutase (E-SOD). There are also two additional isozymes, the copper/zinc-containing SOD (CuZn-SOD) localized in the cytosol and the previously described Mn-SOD, present in the mitochondria [92]. These scavenging proteins have also been found to be upregulated after delayed preconditioning [18, 93]. The effects of SOD in cardioprotection are slightly controversial as it has been shown that neither IPC nor I/R affect its activity in cardiomyocytes [94]. It has been suggested that antioxidant activities may only be altered by episodes of intense myocardial I/R injury [94]. Extracellularly, IPC causes the activation of a SOD-like anti-O₂ \cdot^- mechanism reducing the oxyradical burst [94], and protecting the glycocalyx [26]. Despite the uncertainty regarding the beneficial effect of IPC on SOD levels, other antioxidants have been observed to be upregulated during IPC. This includes mitochondrial uncoupling proteins (UCPs) 2 and 3, where their upregulation is inversely proportional to infarct size in preconditioned hearts [95]. The preconditioned mitochondria were found to produce less hydrogen peroxide compared to control. These UCPs are activated by ROS signaling and are thought to protect the cell from excessive ROS generation in an automatic regulatory forward way [96, 97] which would explain their effectiveness in reducing infarct size in preconditioned hearts. Glutathione (GSH) is an intracellular antioxidant that

scavenges $\cdot\text{OH}$, HOCl, peroxyxynitrate and O₂ \cdot^- radicals during times of oxidative stress [98]. IPC has been found to preserve the levels of GSH in isolated rabbit hearts [13, 98] which partially explains the reduced amount of oxidative damage sustained by preconditioned hearts. Vitamin C, also known as ascorbic acid, is another endogenous antioxidant that, not only has been shown to react with ROS *in vivo*, but is able to remain in cells for long periods [99, 100]. Vitamin C has the ability to protect against H₂O₂-induced cell injury in H9c2 cells [99] and has been shown to be surprisingly effective in protecting plasma lipoproteins from aqueous peroxy radical damage [101]. There is still some debate as to how, or even if, antioxidants play a role in IPC, as literature indicates increases [102], decreases [103, 104], or lack of changes [105, 106], in antioxidant levels. However, it is important to keep in mind that endogenous antioxidants are present in myocardial cells and varying experimental protocols involving different species, methods of inducing IPC and/or I/R injury, and the specific antioxidants studied make this area of research still a mystery waiting to be solved.

ROS CAUSES CARDIOPROTECTION

Although ROS were originally viewed as having only detrimental impacts upon cellular function, there is now evidence that certain levels and types of ROS may be beneficial and possibly contribute to cardioprotection in IPC myocardium. When considering free radicals, there has been an observed "radical threshold" where, below this threshold, cardiac function is able to recover, however above this threshold recovery is not possible [107]. The catecholamine adrenaline (ADR) is known to evoke a pro-oxidant signal which causes the translocation of protective transcription factors HSF-1 and NF κ B. Interestingly, in isolated rat cardiomyocytes, the pro-oxidant signal from ADR also decreases proteasome activity, which can be recovered upon the addition of the ROS scavenger, tiron [108]. It has also been reported that an increase in endogenous ascorbyl free radical formation may improve functional recovery despite its contribution to oxidative stress [109].

Hyperoxia

The utilization of new technology, such as electron paramagnetic resonance (EPR) paired with oxygen sensitive probes, such as LiPc, allow scientists to measure specific PO₂/redox status *in vivo* [110], and Doppler flow measurement allows for the investigation of changes in oxygen consumption and tissue oxygenation *in vivo* [111]. After I/R, myocardial tissue reveals hyperoxygenation [112] suggesting the possibility that I/R causes the myocardium to utilize less oxygen [111]. It has been shown that ROS and reactive nitrogen species (RNS), formed during I/R, inhibited mitochondrial oxygen consumption [112], however, IPC attenuated hyperoxygenation due to observed higher blood flow and lower PO₂ and the possible preservation of higher levels of O₂ utilization [111]. I/R injury was also shown to occur in the brain where the treatment of normobaric hyperoxia (95% O₂ with 5% CO₂) immediately after I/R was found to reduce the infarction volume and improve neurological function close to pre-ischemic levels [113]. It was hypothesized that the addition of O₂ may decrease ROS generation during

ischemia, as there was no increase in oxidative stress when normobaric hyperoxia treatment was applied during focal cerebral I/R [113, 114]. Hyperoxia treatment has also been studied in rat hearts [115, 116]. In one study, mechanically ventilated rats were exposed to hyperoxia for 30 minutes before the hearts were isolated and subjected to 30 min of ischemia followed by 2 hr of reperfusion. Hyperoxia was found to improve cardiovascular function, left ventricular end-diastolic pressure, left ventricular end-developed pressure, and reduce infarct size [115]. Another study reported similar results when inducing hyperoxic preconditioning in addition to reductions in cytochrome-c release and DNA fragmentation which suggest that hyperoxia increases cardiomyocyte tolerance by ROS activation of NF κ B [116]. In an open-chest rabbit model of I/R, the exposure of the heart to 100% oxygen during ischemia-only, reperfusion-only, and I/R resulted in smaller infarct sizes as well [117]. It is thus evident that low concentrations of ROS play a role in IPC that allow for cardioprotection where alterations in the subcellular organelles may not occur to the point where drastic changes occur that impair cardiac function.

Nitric Oxide and Nitric Oxide Synthases

Endogenous NO and NOS - originally thought of as sources of oxidative stress on cardiomyocytes - have properties that contribute to IPC and cardioprotection. The excess O₂⁻ caused by I/R injury is scavenged by NO donors, thus protecting the myocardium from I/R injury [118, 119]. In newborn rat cardiomyocytes, treatment with an NO donor (sodium nitroprusside) can activate SERCA2a causing an increase in Ca²⁺ uptake and subsequently preventing cytosolic Ca²⁺ upload [120]. NO can also contribute to the S-nitrosation whose primary target is Complex I of the electron transport chain in the mitochondria, causing its decrease in activity which occurs exclusively in I/R [121]. The modification of cysteine residues by NO \cdot has been hypothesized to form a "molecular cap" that prevents further oxidation of thiols by ROS, resulting in the regulation of ROS formation from mitochondria and caspase-3 apoptotic activity [122-124]. NOS has been implicated in preconditioning and cardioprotection, as it was observed to decrease infarct size and increase the recovery of left ventricular diastolic pressure; there was a four-fold increase of Hsp90 association with eNOS and increases in eNOS itself in the myocardium [125]. eNOS has demonstrated possessing cardioprotective properties and its involvement in preconditioning has been shown, in a variety of studies, to recruit endothelial progenitor cells (EPC) that express potentially cardioprotective cytokines including NOS isoforms [126]. The complex formed between eNOS and HSP90 allows for phosphorylation enhancement of eNOS *via* Akt, amplifies NO release, and increases production of cyclic guanosine monophosphate [127-129]. Protection has been implied as being partially dependent on this HSP90/eNOS association enhancement and the resulting NO release [130]. IPC has been shown to preserve the function eNOS as well, hastening endothelial recovery and function [131].

CONCLUSION

In I/R, oxidative stress leads to cardiac dysfunction which can be attenuated by IPC. ROS, the cause of oxidative

stress, alter the functions of various subcellular organelles in both I/R injury and IPC [12]. The nucleus is shown to upregulate a slew of cardioprotective genes that have shown effects in late-phase IPC [17, 25], largely as a result of increased NF κ B action [17]. IPC reduces the effects of MMP-2 ultimately protecting the glycocalyx and increases the stability of contractile myofilaments [27, 30-32]. SR, the principal Ca²⁺ regulator of the cell responsible for maintaining contractile function, undergoes alterations of its Ca²⁺ channels, including SERCA, NCX, and RyRs [36, 39, 41] which are modified in I/R injury and preserved by IPC. Oxidative stress has also shown to have an inhibitory effect on SL Na⁺-K⁺-ATPase [43-45] and to physically disrupt the phospholipid bilayer [9]. With regards to IPC, the most effective protection of the SL is the activation of sarc K⁺-ATP channels, however, the extent of its effect compared to mito K⁺-ATP channels is still a debatable topic. Both these K⁺-ATP channels have been shown to be effective in different animal models where the mitochondrial isoform acts as an effector and a trigger, and the SL isoform is an effector [55]. The importance of the SL isoform is emphasized by the studies of Kir 6.2 KO mice where the missing pore subunit of the sarc K⁺-ATP channel prevented protection of I/R injury post-IPC [56]. Mitochondria are the main source of free radicals in the cell, and, coupled with their "ROS-induced ROS release" mechanism, are not only affected by oxidative stress but incur further damage upon the cell [5, 10]. IPC has been shown to inhibit the electron transport chain, particularly at the electron entry point of Complex I [67, 68], suggesting that a low amount of ROS triggers an anti-apoptotic protective measure whereas high amounts of ROS promote mitochondrial release of pro-apoptotic factors for the development of oxidative stress.

Several endogenous protective interventions have also been demonstrated to be activated when triggered by IPC. These include the upregulation of COX-2 [76] and activation of the PKC ϵ pathway [82, 83]. Interestingly, upon low concentrations of oxidant exposure, PLD signals for cell survival are generated during IPC [85]. Thioredoxin, a survival signal transmitter active in ischemic myocardium, also has increased activity in IPC [90]. Despite the existing debate regarding the effectiveness of different antioxidants in IPC, eSOD [26, 92], mitochondrial UCPs 2 and 3 [95], GSH [98], and vitamin C [101] still seem to be involved. Although these antioxidants become more active, there is still need for further exploration on their effects. Oxidative stress itself has demonstrated cardioprotective properties and involvement in IPC. The pro-oxidant signal of ADR increases the translocation of protective transcription factors [108], whereas hyperoxia has also been shown to be protective in both rat and rabbit models of IPC [115-117]. In IPC and cardioprotection NOS, especially eNOS, has shown effectiveness in attenuating oxidative damage, particularly when it forms a complex with HSP90 [125-129]. An outline of proposed pathways differentiating the two phases of preconditioning is displayed in Fig. (2). Most of the work on IPC showing beneficial effects for the recovery of cardiac function preceding I/R injury has been carried out in the isolated heart preparation. However, this procedure should be extended to the clinical setting where IPC should be conducted by 3 to 5 cycles of

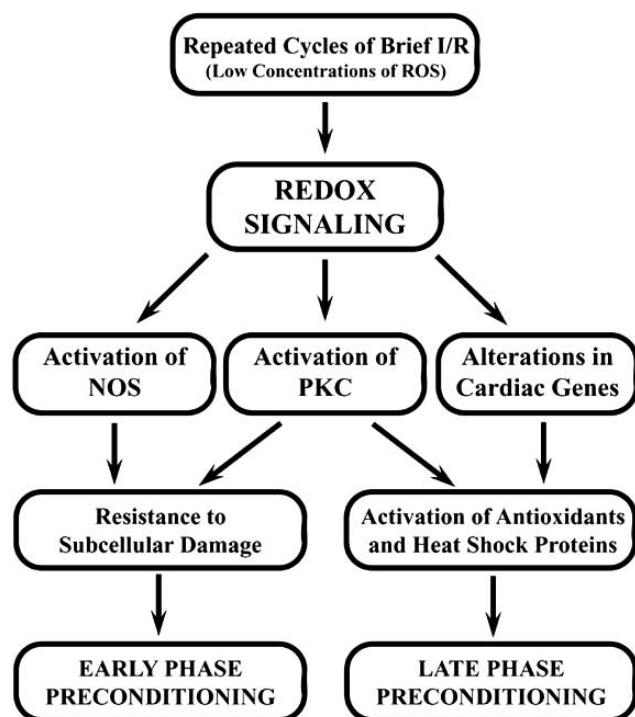


Fig. (2). Outline of proposed pathways of redox induced early and late phases of ischemic preconditioning. Abbreviations: I/R, ischemia-reperfusion; ROS, reactive oxygen species; NOS, nitric oxide synthase; PKC, protein kinase C.

clamping and releasing of the aorta before carrying out coronary bypass surgery, angioplasty, heart transplantation, or thrombolytic therapy. IPC can cause resistance to oxidative damage by altering the redox state of cardiomyocytes and can be protective by increasing the resistance of subcellular organelles to ROS modifications.

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