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REVIEW

Ischemic and hypoxic preconditioning protect cardiac muscles via intracellular ROS signaling

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Abstract **Oxidative stress can cause extensive damage to cardiac tissue under reperfusion conditions. However, preconditioning the myocardium may diminish these negative effects and alleviate reperfusion injury. There are a variety of preconditioning therapies, such as ischemic preconditioning (IPC) and hypoxic preconditioning (HPC), each targeting specific channels, receptors, and/or intracellular molecules. Ischemic preconditioning involves brief periods of ischemia followed by brief periods of reperfusion, thus strengthening the cardiac resistance for a longer period of ischemia. IPC involves complex mechanisms, some of which are still not completely understood today. Nevertheless, many studies have already established models of IPC. In addition, similar to IPC, HPC has also been recognized as preventing reperfusion injury. Reactive oxygen species (ROS) are known mediators of IPC and HPC. Particularly, mitochondria-generated ROS initiate activity of several beneficial preconditioning pathways. The role of ROS is paradoxical; low levels of ROS are key factors in signaling IPC/HPC, but high levels of ROS can contribute to increased oxidative stress on cardiomyocytes. Therefore, it is important to determine the molecular mechanism of IPC and HPC to avoid excessive accumulation of ROS to prevent cardiac injury. In this review, we will outline IPC and HPC, explaining the putative role of ROS in both pathways. We will also discuss preconditioning efficacy in certain conditions such as exercise and how the aging myocardium responds to preconditioning therapies.**

Keywords hypoxia, ischemia-reperfusion, ROS, cardiomyocyte, preconditioning

Introduction of ischemic preconditioning

Cardiologists observed a beneficial "warm-up" effect in those patients suffering acute but short ventricle infarctions resulting in less cardiac dysfunction and angina over time. This raised a paradoxical debate on the increased time of total ischemia, including the acute and chronic periods (Jaffe and Quinn, 1980). This acute "warm-up," defined as ischemic preconditioning (IPC), includes an extended period of energy supply and a delayed cellular necrosis after a long period of ischemia. IPC induces cardioprotection that includes both irreversible cardiac injuries and chronic myocardial remodeling (Kloner and Jennings, 2001).

Ischemic injury results from the loss or restriction of significant blood and oxygen supply to myocardium. Reperfusion injury occurs when blood supply returns to the coronary system after a certain period of ischemia, causing structural damage to cardiac muscle (Braunwald and Kloner, 1985; Nayler and Elz, 1986). Since the oxygen demand can only be met by aerobic metabolism, when the myocardium is suddenly exposed to ischemia, coronary arterioles and resistant vessels dilate to increase blood flow up to three to five times above basal levels to supply as much oxygen as possible to the heart. During a short period of ischemia, such as the acute phase, this allows the coronary arteries to be "exercised" with increased cardioprotection. However, due to the lack of anaerobic pathways, eventually the absence of oxygen in a longer period of ischemia reduces ATP storage, causing the cardiac muscles to become vulnerable to ischemia (Sanada et al., 2011). Because the myocardium consumes a large amount of ATP, the usage of ATP and other high energy phosphates are important in maintaining cellular homeostasis, particularly during ischemia. The reduced ATP storage due to long periods of ischemia, therefore, causes the cardiac muscles to become vulnerable to damage. IPC, however, can decrease these damages by delaying ATP consumption

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(Sanada et al., 2011).

Furthermore, previous experiments on animals show that a series of brief ischemia and reperfusion cycles significantly limits injury to the mitochondria caused by a prolonged period of ischemia and reperfusion (I/R) (Yang et al., 2010). In a study conducted by Murry et al., prior to ischemia, canine coronary arteries were exposed to four brief periods of 5 min of ischemia, followed by 5 min of reperfusion. After 40 min of ischemia, the cardiac infarct size was reduced to only 25% of that in the control group (Yang et al., 2010). This intervention demonstrates an example of IPC protection. Further research has revealed that IPC is composed of two critically time related phases of protection: early phase and delayed phase. The early phase of IPC lasts for a short period of time such as 1 h. The delayed phase appears 24 h after the early phase and can last for up to 3 days (Pagliaro et al. 2001).

Role of IPC and ROS in ischemia/reperfusion

Reactive oxygen species (ROS) are believed to play an essential role in ischemic preconditioning. Organelles in the heart that are affected by IPC include the sarcolemma, mitochondria, sarcoplasmic reticulum, myofibrils, glycocalyx, and the nucleus (Saini et al., 2004). ROS are generated during myocardial reperfusion from functional mitochondria in cardiomyocytes and from activated xanthine oxidase in vascular endothelium (Opie, 1992). Excessive amounts of ROS cause cardiac cell damage and postischemic contractile dysfunction (Dhalla et al., 2000a). This is because potent ROS radicals, such as hydroxyl ions, can effectively attack all cellular structures, enzymes, and protein channels, resulting in degradation of intracellular proteins, rupture of cellular membranes, intracellular Ca^{2+} overload, cellular necrosis, and apoptosis (Sanada et al., 2011). However, low amounts of ROS produced during brief periods/cycles of I/R exert the beneficiary effect of IPC (Osada et al., 1994; Tanaka et al., 1994). For example, mitochondria from I/R preconditioned cardiac muscle generate less ROS after prolonged I/R compared to the control group, causing less oxidative stress (Park et al., 1997; Dhalla et al., 2000b). The cardioprotection mechanism of IPC involves activating multiple molecular signaling pathways, and is discussed in the next section.

Molecular mechanisms of IPC protection via sarcolemmal and mitochondrial K_{ATP}

Sarcolemmal ATP potassium channels (sarc K_{ATP}) play an important role in reducing ischemic injury through IPC (Suzuki et al., 2002). This mechanism is related to norepinephrine release from cardiomyocytes during ischemia (Abrahamsson et al., 1985; Carlsson et al., 1985). Norepinephrine binds to an adrenergic α_1 receptor to initiate a cascade of intracellular protein activation, and this process may increase the activity of sarc K_{ATP} channels (Turrell et al., 2011), resulting in the hyperpolarization of the plasma membrane potential. During I/R, increased intracellular Ca^{2+} concentration can potentially activate both proteases and phospholipases that decompose membrane proteins and phospholipids causing permanent impairment of the membrane integrity (Atsma et al., 1995). Therefore, the Ca^{2+} entry is limited, and damages by Ca^{2+} overload during I/R are eventually prevented during the IPC induced hyperpolarization mentioned above (Behling and Malone, 1995; Light et al., 1996). In addition, sarc K_{ATP} channels have been shown to be physically linked to several key metabolic enzymes, such as adenylate kinase (Crawford et al., 2002) and creatine kinase (Carrasco et al., 2001). The opening of sarc K_{ATP} channels may activate phosphate energy transfer by adenylate kinase and creatine kinase, leading to the dramatic increase of ATP generation and consequently improved IPC effects during ischemia (Turrell et al., 2011).

IPC also partially inhibits the activity of mitochondria. Particularly, complex II (succinate dehydrogenase) inhibition by IPC opens mitochondria (mito) K_{ATP} channels to allow cytosolic K^+ to enter the mitochondria (Wojtovich and Brookes, 2008). This causes the subsequent activation of K^+/H^+ antiporters that contribute to matrix swelling (MS) (Garlid et al., 1996) which has been shown to enhance fatty acid oxidation and ATP synthesis leading to cardio-protective effects (Halestrap, 1989; Fryer et al., 2000), as seen in Fig. 1. IPC inhibition of mitochondria results in ROS generation (Ambrosio et al., 1993; Zuo et al., 2003). Gross et al. found that IPC efficacy is related to the initial burst of ROS followed by the activation of both sarc K_{ATP} and mito K_{ATP}, and that antioxidants completely abolish this cardioprotection (Gross et al., 2007). Therefore, ROS play a critical role in signaling IPC.

IPC protects Na^+/K^+ ATPase

Na^+/K^+ ATPase plays a vital role in maintaining resting membrane potentials in cardiomyocytes especially since the inactivity of this enzyme results in immediate cell dysfunction or death (Elmoselhi et al., 2003). During I/R, both the mRNA level and enzymatic activities of Na^+/K^+ ATPase are largely reduced, thus resulting in heart injuries (Kim and Akera, 1987; Elmoselhi et al., 2003). However, when the cells are subjected to IPC, these negative effects exerted by I/R are significantly attenuated and heart function is preserved (Elmoselhi et al., 2003). Moreover, the Na^+/Ca^{2+} exchanger, another important Ca^{2+} pump to prevent cell death due to Ca^{2+} overload, is also protected by IPC during I/R in a similar mechanism (Saini et al., 2004). It is widely believed that ROS formation and oxidative damages occur during I/R (Kim and Akera, 1987; Zuo et al., 2009), therefore, IPC protection of Na^+/K^+ ATPase involves ROS induced signaling cascades that trigger intracellular antioxidant defense mechanisms to reduce the damage of the ion pumps (Saini et al., 2004).

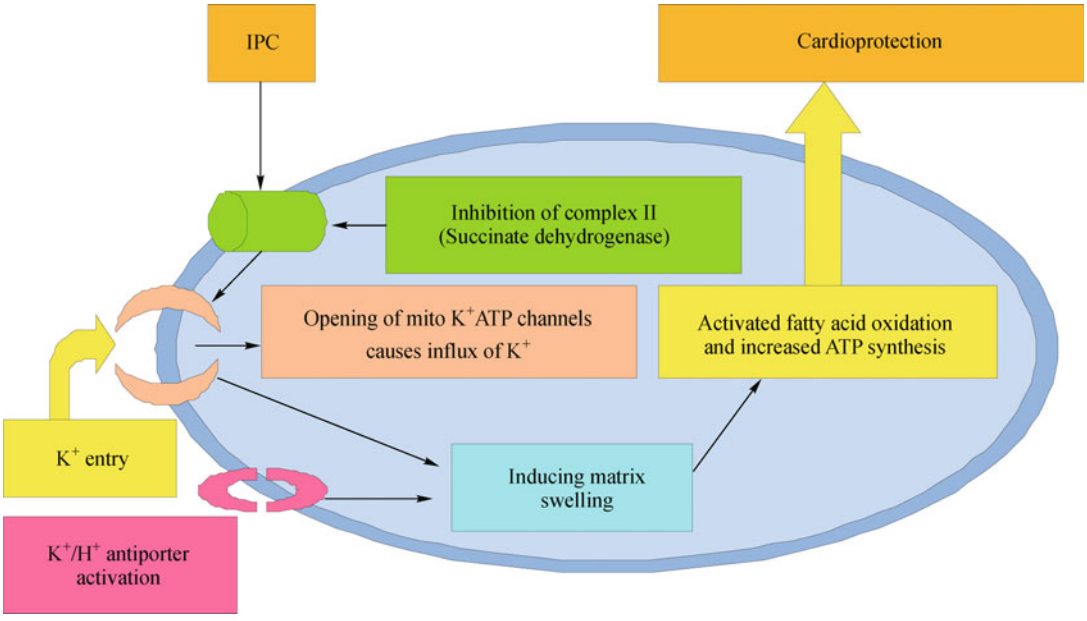

Figure 1 This schematic demonstrates an intracellular pathway induced by ischemic preconditioning (IPC) during I/R.

Role of preconditioning in hypoxia

Oxygen plays an important role in the biologic system. Particularly, the heart is an aerobic organ requiring a large amount of oxygen supply to function properly. Thus, oxygen is essential for the sustainability of myocardial contractility and contractile tension (Davies, 1995). However, the role of oxygen in the mammalian heart is complicated, and involves multiple molecular metabolisms associated with energy usage (Giordano, 2005). Lack of oxygen initiates the myocardial hypoxia inducible factor-1α (HIF-1α) gene expression to coordinate limited oxygen support in the heart, while the presence of oxygen inhibits this gene transcription (Huang et al., 2004). Oxygen also regulates the vascular tone by inducing NO formation (Davies, 1995). It is worth noting that oxygen is a "double-edged sword" molecule since oxygen is a central source of ROS formation. Excessive ROS can cause deleterious effects on the biologic system such as cancer, myocardial dysfunction, DNA mutation, and other cellular damages (Davies, 1995).

Hypoxia occurs when a region of the body is deprived of an adequate amount of oxygen. During hypoxia, all nutrient supply is maintained except the reduced oxygen level. This is significantly different from ischemia during which both oxygen and nutrient supply are restricted. Therefore, hypoxia stress seems a little "mild" compared to ischemia, thus the preconditioning mechanism of hypoxia may be different. However, IPC significantly increases the activities of major antioxidant enzymes including superoxide dismutase (SOD) and catalase (Yuan et al., 2005). Since antioxidant enzymes are the key factors involved in cellular defense mechanisms against hypoxia induced stress, HPC can enhance the expression of intracellular SOD levels (Chen et al., 2003). Thus, both IPC and HPC seem to share similar redox pathways responding to oxidative stress caused by limited oxygen supply. Moreover, previous research has shown HIF-1α is required for IPC-induced mitochondrial ROS production (Cai et al., 2008). Although the underlying mechanism is not entirely understood, it appears that HIF-1α plays an essential role in triggering cellular protection in both IPC and HPC via ROS signaling.

In cardiomyocytes, a short hypoxic exposure induces a small amount of ROS generation that plays an essential role in reducing cell death caused by subsequent prolonged hypoxia (Vanden Hoek et al., 1998). This is called hypoxic preconditioning (HPC) protection, which is consistent with previous studies showing that low levels of ROS stimulate intracellular signaling cascades by increasing ATP utilization and myocyte contraction (Duranteau et al., 1998). Furthermore, HPC stimulates angiogenesis and adipose-derived stem cell activity to reduce ischemic injuries (Stubbs et al., 2012). The molecular mechanism of HPC protection involves ROS-mediated multiple signaling pathways as discussed below.

Molecular mechanism of HPC protection via ROS

Vanden Hoek et al. suggested that HPC induced ROS (mainly H_2O_2) provide cardio protection against I/R injuries (Vanden Hoek et al., 1998). This ROS centered preconditioning may be mediated by Protein Kinase C (PKC) activity (Gopalakrishna and Anderson, 1989) (Fig. 2). During HPC, mitochondria generate superoxide ions that eventually exit the mitochondria via anion channels to the cytosol where it is converted to H_2O_2 by SOD (Vanden Hoek et al., 1998), which

can also be regulated by HIF-1α (Chen et al., 2003). Cytosolic H_2O_2 has been shown to activate PKC (Vanden Hoek et al., 1998). During early reperfusion, PKC activates adenosine receptors causing PI3-K activation (Downey et al., 2008). PI3-K further induces ERK which inactivates GSK-3β, thus inhibiting the opening of mPTP (Juhaszova et al. 2004). This is very important for the cell survival during I/R since the opening of mPTP channels in non-HPC conditions may negatively disrupt the proton electrochemical gradient, inhibit ATP synthesis, and induce cellular organelle rupture (Yang et al., 2010).

Preconditioning induced by exercise

Elevated levels of ROS produced in skeletal muscle during heavy physical exercise are associated with muscle dysfunction (Peternelj and Coombes, 2011). However, preconditioning induced by mild or regular exercise, has been shown to protect hearts from potential I/R injuries by decreasing cardiac infarctions, increasing mitochondria functions, and boosting heat shock protein activity and antioxidant defense system (Ascensão et al., 2007). Consistently, in animal models, Hamiton et al. found that antioxidant enriched myocardial tissues in rats have sustained smaller infarctions (Hamilton et al., 2003). Therefore, exercise induced preconditioning provides marked cardioprotection during I/R via ROS. In addition, previous research has identified a gene regulating haem oxygenase-1 is responsible for ROS signaling involved in exercise induced preconditioning (McArdle et al., 2004).

Effect of aging on IPC

Aging is initiated by free radicals, causing gradual damages to biologic molecules (Rose et al., 2011). Uncoupling proteins (UCPs) are carriers located in the mitochondrial inner membrane that have a tremendous impact on aging (Rose et al., 2011). It has been shown that UCPs maintain balanced levels of ROS and stabilize biologic homeostasis, therefore increasing longevity (Rose et al., 2011). During I/R, aged cardiomyoctes tend to have more myocardial infarctions, even with IPC treatment (Wojtovich et al., 2012), and thus become more resistant to IPC (Bélichard et al., 1987). For instance, IPC in aged rats neither enhances recovery nor increases postischemic function (Sanada et al., 2011). This could be due to the interruption of redox status, Ca^{2+} homeostasis and mitochondrial oxidative phosphorylation associated with aging (Wojtovich et al., 2012). In human studies, there is no reduced infarct size after IPC treatment in patients over 65 years old (Abete et al., 1997). Obviously, the increased oxidative stress associated with aging completely abolishes any effect of preconditioning (Hekimi et al., 2011)

Furthermore, altered gene expression in aged myocardium also contributes to diminished cardioprotection from IPC. For

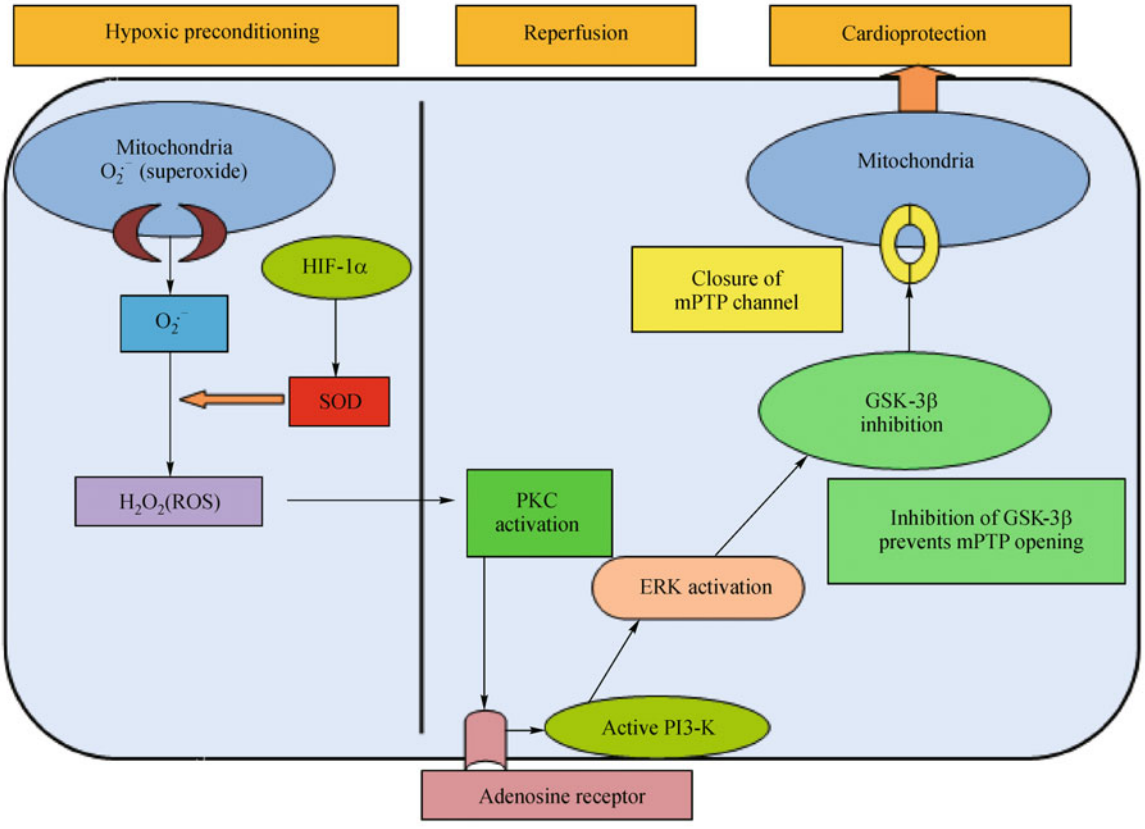

Figure 2 This schematic demonstrates a putative intracellular pathway induced by hypoxic preconditioning (HPC) during I/R.

example, aged cardiomyocytes show reduced expression of some of the key IPC signaling molecules including PCK–ε, ERK and Akt, as well as antioxidant enzymes such as catalase and MnSOD (Boengler et al., 2009). Since activation of these molecules during IPC provides beneficial effects such as reducing oxidative stress by endogenous catalase and MnSOD, this decrease may disrupt the preconditioning efficacy and consistency. In addition, the opening of the mitochondrial permeability transition pore (mPTP) in aged myocardium contributes to increased ROS generation and initiation of cell apoptosis pathways (Brookes et al., 2004; Murphy and Steenbergen, 2007). Thus the loss of IPC protection and stimulation of mPTP may cause permanent damage or death in aged cells during I/R (Lemasters et al., 2009).

Conclusions

In this review, we have discussed the current findings regarding molecular mechanisms of IPC and HPC against ischemic and hypoxic injuries on cardiac muscles as well as the effect of aging and exercise on preconditioning. Based on both animal and human experiments, we have summarized the ongoing investigations of the protective pathways involved in these oxygen related injuries. Although the complex mechanism of precondition is not completely understood, we propose that ROS play a fundamental role in both IPC and HPC signaling cascades.

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