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Cardioprotection Techniques: Preconditioning, Postconditioning and Remote Con-ditioning (Basic Science)

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Abstract: Ischemic heart disease (IHD) is the leading cause of death and disability worldwide. The major pathological consequences of IHD arise from the detrimental effects of acute ischemia-reperfusion injury (IRI) on the myocardium. Therefore, in order to improve clinical outcomes in patients with IHD, novel therapeutic strategies are required to protect the myocardium from acute IRI and preserve cardiac function (cardioprotection). In this regard, endogenous cardioprotective strategies such as ischemic preconditioning (IPC), ischemic postconditioning (IPost) and remote ischemic conditioning (RIC) may provide novel approaches for protecting the heart in clinical settings in which the patient experiences acute myocardial IRI. In this review article, we provide an overview of these endogenous cardioprotective strategies with respect to the pre-clinical experimental literature, exploring their major characteristics and underlying signaling mechanisms. The application of these therapeutic strategies in the clinical setting for potential patient benefit is reviewed in another article in this special issue.

Keywords: Ischemic preconditioning, ischemic postconditioning, remote ischemic conditioning, cardioprotection, ischemia-reperfusion injury.

1. INTRODUCTION

"…..we could exploit ischemia to protect the heart from ischemic injury." Murry *et al* 1986 [1].

Ischemic heart disease (IHD) is the leading cause of death and disability worldwide. Despite optimal therapy, the morbidity and mortality from IHD remain significant. As such, novel therapeutic strategies are required to protect the heart from the detrimental effects of acute ischemia-reperfusion injury (IRI), in order to reduce myocardial injury, preserve cardiac function and improve clinical outcomes in patients with IHD.

Over 25 years ago, Murry and co-workers [1] first introduced the possibility of harnessing the heart's own ability to protect itself from acute lethal IRI by preconditioning it with brief non-lethal episodes of ischemia and reperfusion, an endogenous cardioprotective phenomenon which was termed ischemic preconditioning (IPC). In that landmark experimental study, four-5 min cycles of alternating left anterior descending (LAD) coronary artery occlusion and reflow applied immediately prior to a 90 min LAD occlusion and 3 day reperfusion period, reduced myocardial infarct (MI) size in the canine heart to 25% of that observed in untreated control hearts. The inspiration for undertaking this seminal study was based on the results of experimental studies which had unexpectedly found that repeated bouts of myocardial ischemia and reperfusion did not cause a cumulative detrimental effect on myocardial ATP content, cell death and myocardial function [2, 3].

IPC remains one of the most powerful therapeutic strategies for reducing MI size and has become a ubiquitous phenomenon, protecting every species it has been tested in including man. Despite intensive investigation and the publication of nearly 7,000 studies on PubMed, the on-going challenge has been to elucidate the mechanistic pathways underlying IPC. Over the years the concept of IPC has evolved to include ischemic postconditioning (IPost) and remote ischemic conditioning (RIC), therapeutic strategies which can be collectively termed ischemic conditioning. These advances have greatly facilitated the translation of ischemic conditioning into the clinical settings of acute IRI. The clinical application of ischemic conditioning is discussed in another article in this special issue. In this review article, an overview will be provided of these 3 forms of ischemic conditioning, with respect to the pre-clinical experimental literature exploring their major characteristics and underlying signalling mechanisms. It would be impossible for a single review article to provide a comprehensive account of these individual endogenous cardioprotective phenomena and for this the reader is referred to the following review articles [4-7].

2. HOW THE CONCEPT OF IPC HAS EVOLVED OVER THE YEARS

The concept of IPC has evolved over the years since its initial discovery in 1986 to now include remote ischemic conditioning (RIC) and ischemic postconditioning (IPost). The first major development took place in 1993 by Przyklenk *et al* [8] who found that applying an IPC protocol in one coronary vascular territory could actually protect the myocardium in a different coronary vascular territory from a subsequent lethal episode of acute myocardial IRI. In this landmark experimental study undertaken in canine hearts, IPC in the circumflex artery (four-5 min alternating cycles of occlusion and reflow), immediately prior to a 60 min LAD occlusion and 3 day reperfusion period, reduced MI size to 38% of that observed in untreated control hearts [8]. The idea of undertaking this experiment was based on the findings of a study by the same research group in isolated rat hearts, in which it was demonstrated that MI size expressed as a percentage of the area-at-risk actually increased in IPC-treated hearts whereas in control hearts, MI size did not increase [9]. This led the authors to propose that IPC might somehow produce a diffusible protective factor in remote non-ischemic myocardium [9]. This intramyocardial preconditioning effect between two different but adjacent coronary vascular beds was further developed in an experimental study in 1996, demonstrating that the IPC stimulus could actually be applied to an organ away from the heart. Gho *et al* [10] demonstrated that applying 15 min of anterior mesenteric artery occlusion followed by 15 min reflow to induce a brief period of episode of intestinal ischemia and reperfusion immediately prior to 60 min coronary artery occlusion and 180 min reflow reduced subsequent myocardial infarct size by 28%. This phenomenon has been termed remote ischemic preconditioning (RIPC), and subsequent studies have demonstrated that the precon-

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ditioning stimulus can be applied in any organ or tissue to protect any other organ or tissue from a sustained lethal episode of acute IRI- termed inter-organ preconditioning [7]. Crucially, the remote preconditioning stimulus has been shown to be effective if applied prior to (RIPC) [10], after the onset of (remote ischemic preconditioning) [11] and even at the onset of myocardial reperfusion (remote ischemic postconditioning) [12]. Furthermore, the ability to remotely precondition the upper or lower limb to protect the heart from acute IRI has greatly facilitated the translation of remote ischemic preconditioning into the clinical setting of cardiac surgery, acute myocardial infarction and coronary angioplasty [13-16].

The next major development occurred soon after the first description of RIPC, with the discovery in 1993 by Kuzuya *et al* [17] that IPC actually induced two distinct windows of cardioprotection. These authors demonstrated in the canine heart that following a standard IPC stimulus, MI size reduction was observed immediately but was lost 6 or 12 hours later and then re-appeared again at 24 hours and lasted until 72 hours [17]. This delayed effect of IPC has been termed the second window of protection or delayed or late preconditioning [18].

In 2003, Zhao *et al* [19] found that by simply interrupting myocardial reperfusion with several short-lived episodes of myocardial ischemia could reduce MI size to a level comparable to IPC. These authors demonstrated in canine hearts that following a 60 min of coronary artery occlusion, by allowing reflow for 30 seconds and then re-occluding the coronary artery for 30 seconds a cycle which was repeated a total of 3 times, reduced MI size when compared to hearts which received uninterrupted reperfusion [19]. This phenomenon, which has been termed ischemic postconditioning (IPost) provides a cardioprotective therapeutic strategy which can be applied at the onset of myocardial reperfusion, thereby allowing its rapid translation into the clinical setting in patients presenting with an acute myocardial infarction [20] and in patients undergoing cardiac surgery [21]. The elucidation of the mechanisms underlying these different forms of ischemic conditioning has identified novel targets for cardioprotection amenable to pharmacological manipulation (so-called pharmacological conditioning).

3. ISCHEMIC PRECONDITIONING

3.1. The IPC Stimulus

In the original study by Murry *et al* [1], four-5 min cycles of myocardial ischemia and reperfusion were chosen to precondition the canine heart against myocardial infarction. This treatment protocol was chosen by the authors as longer periods of myocardial ischemia (such as 10 or 15 min) resulted in arrhythmias and increased mortality [1]. The stated reason for using four cycles was to increase the formation and wash-out of ischemic catabolites [1]. The 5 min reperfusion period was chosen to be long enough to wash-out lactate and re-accumulate high-energy phosphates [1]. A wide variety of stimuli have been shown to induce a preconditioning-like effect in terms of cardioprotection such as heat stress, hypothermia, exercise, pacing, myocardial stretch and so forth.

A standard IPC stimulus induces 2 phases of cardioprotection: the first phase (termed classical preconditioning or the first window of protection) begins immediately following the IPC stimulus and lasts for 2-3 hours, after which the cardioprotective effect wanes and disappears, and a second phase (termed delayed or late preconditioning or the second window of cardioprotection) re-appearing 12-24 hours later and lasting up to 72 hours [18].

3.2. The Signaling Pathways Underlying IPC

The intracellular signalling pathways underlying ischemic conditioning in the heart are numerous and remarkably complex and as such, they cannot be comprehensively reviewed in a single article. For IPC, the current paradigm suggests that the cardiomyocyte generates autacoids (such as adenosine, bradykinin, endothelin, opioids) in response to the IPC stimulus which bind to their respective G-protein coupled receptors and activate a number of signalling pathways, many of which appear to converge on the mitochondria (see (Fig. **1**) for a simplified overview). In IPC, the signal transduction pathway can be conceptually classified into triggers (these are components of the signaling pathway which mainly act before the index ischemic episode and activate downstream signaling mediators) or mediators and end-effectors (these are components of the signaling pathway which act during the index ischemic episode or at the time of reperfusion and mediate the protective effect). However, this separation is not rigid as certain signaling components have been demonstrated to act as both triggers and mediators/effectors.

3.2.1. Triggers of IPC

In 1991, Liu *et al* [22] first implicated the adenosine A1 receptor as a trigger of IPC, by demonstrating that it could be blocked by the non-specific adenosine receptor blocker, 8-sulfophenyltheophylline (8-SPT), and that the intracoronary administration of either adenosine or an adenosine A1 receptor agonist could reduce MI size. These important findings demonstrated that IPC was a receptor-mediated phenomenon and suggested that the infarct-limiting effects of IPC could be mimicked by a pharmacological agent [22]. Other GPCR ligands have been implicated as triggers of IPC such as bradykinin [23, 24], opioids [25], acetylcholine [26], catecholamines [27], angiotensin II [28], and endothelin-1 [29]. The IPC triggers are highly redundant with multiple cycles of IPC able to overcome the effect of antagonism at a single receptor [24]. The simultaneous activation of these GPCRs during the IPC stimulus suggested that the cardioprotective signal probably converges on a single downstream mediator, protein kinase C (PKC), which mediates the memory effect of IPC. Other triggers of IPC, which are dealt with in later sections, include the MitoK_{ATP} channel, reactive oxygen species (ROS) and nitric oxide.

3.2.2. Mediators of IPC

The downstream mediators of IPC are mainly protein kinases such as PKC, tyrosine kinase, Akt, and the mitogen activated protein kinases (MAPK) which are activated by the triggers of IPC. Experimental studies by Ytrehus *et al* in 1994 [30] showed that IPC could be blocked a non-specific antagonist of PKC and reproduced by PKC analogues. Although there are some differences with species, IPC has been reported to activate the PKC-ε isoform as a mediator of cardioprotection, whereas it has shown to inhibit the activation of the PKC-δ isoform, which has been reported to have detrimental effects in the setting of IRI. It has been suggested that the PKC-ε isoform may play a key role in the mitochondria. In 1996, Maulik *et al* [31] were the first to demonstrate that genistein, a tyrosine kinase antagonist could block IPC in rat hearts. Receptor tyrosine kinases may act as IPC triggers by activating PKC and cytosolic tyrosine kinases may act as IPC mediators by acting either downstream or in parallel with PKC [32].

Experimental studies have implicated signaling through the phosphatidyl inositol 3-OH kinase (PI3K)-Akt cascade during the preconditioning phase before the index ischemic episode [33] and at the time of myocardial reperfusion as part of the Reperfusion Injury Salvage Kinase (RISK) pathway [34]. In IPC Tong and colleagues [33] were the first to demonstrate that IPC activates the PI3K-Akt kinase cascade prior to the index ischaemic episode, and that the PI3K inhibitor Wortmannin could abolish IPC. We showed that IPC could activate Akt at the time of myocardial reperfusion and that pharmacologically inhibiting PI3K at this time could block IPC protection, suggesting that IPC could modify signalling events happening at the time of myocardial reperfusion [34]. Solenkova *et al* [35] extended these findings by demonstrating that the IPC induced activation of Akt could be abrogated by 8-SPT (an adenosine receptor antagonist), suggesting that endogenous adenosine activated Akt in preconditioned hearts.

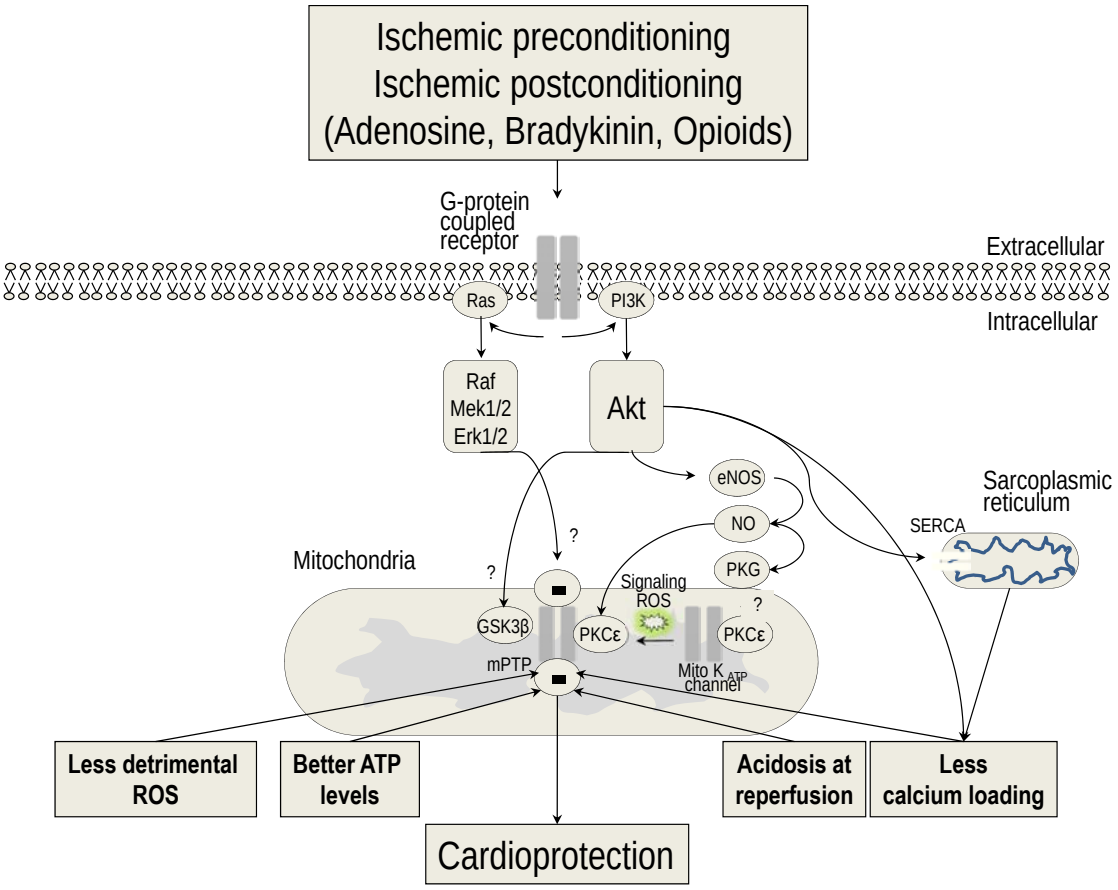

Fig. (1). Signaling pathways linking IPC and IPost to the mPTP. This scheme provides a simplified overview of two of the major signaling pathways implicated in both IPC and IPost which link the cell-surface receptor to the mPTP. Of course many other signalling pathways have been implicated in both IPC and IPost but for the purposes of clarity these have not been included in this scheme. IPC and IPost are both seen to activate cell surface receptor, which then recruit a number of signal transduction pathways including the PI3K-Akt and MEK1/2-Erk1/2 pathways which terminate on the mitochondria with the activation of the MitoK_{ATP} channel and the inhibition of the mPTP. (Figure adapted from [150]).

Whether Erk1/2 MAPK contributes to cardio-protection associated with classical preconditioning is unclear with studies demonstrating that it is activated prior to the index ischaemic period [36-39], but only some of these studies show these MAPK's contributing to IPC-induced protection [36, 37]. In contrast there are studies reporting no change in Erk1/2 activity in the setting of IPC [40, 41]. A study suggests that diazoxide-induced mitochondrial ROS release may activate Erk1/2 [42]. This is interesting given the findings of Baines and colleagues demonstrating that PKC-ε modules can form complexes with Erk1/2-BAD, p38 and JNK at the mitochondria [43]. In this scenario, PKC-ε appeared to phosphorylate both Erk1/2 and p38 but down-regulate JNK [43]. Erk1/2 can mediate cellular protection by phosphorylating recruiting several anti-apoptotic mechanisms.

The role of p38 MAPK in the setting of cardioprotection has been surrounded by controversy and has been the topic of several reviews [44, 45]. There is general agreement that ischemia-reperfusion activates p38 MAPK [46], but the studies investigating the role of p38 MAPK in cardioprotection have attracted much controversy. Experimental Studies suggest that IPC can activate [47-49] or reduce [39, 50, 51] p38 MAPK during the sustained ischemic episode. The generally accepted view is that IPC transiently activates p38 MAPK during the preconditioning phase [52-54], and reduces the p38 MAPK activation that occurs during the sustained ischemic phase [50, 54-56]. Studies have suggested that the p38α-isoform may mediate cell death and the p38β-isoform may contribute to cell survival [57]. In line with this, studies have suggested that it is the p38α isoform which is increased during ischemia [39, 52], and that hypoxic preconditioning protects myocytes by reducing the activation of this isoform during hypoxia [39]. Conversely, Schulz and co-workers [58] found p38β MAPK activity to be increased in preconditioned swine hearts.

Experimental studies have demonstrated JNK activation in the setting of ischemia-reperfusion [40, 46, 52, 59]. The role of JNK in the setting of IPC has been controversial with studies, suggesting both a protective and detrimental aspect to JNK activation. Studies have demonstrated JNK activation in response to a preconditioning stimulus [40, 52, 60, 61] and that JNK mediates the protective effect of IPC [53]. Other reports have found JNK activation in response to a preconditioning stimulus but have failed to find it contributing to protection [62]. Yet other studies have failed to find activation of JNK in the setting of IPC [63]. Unfortunately, a recently published study examining the effect of JNK1/2 transgenic knock-outs has only served to complicate matters further [64]. In this study transgenic JNK knockout mice were found to be protected *in vivo* against myocardial ischemia-reperfusion injury. However, transgenic mice with over-active MKK7, the MAPK kinase upstream of JNK1/2 also paradoxically displayed resistance to myocardial ischemia-reperfusion injury, as evidenced by a reduced infarct size [64].

3.2.3. Effectors of IPC

A number of different effectors have been proposed over the years but most evidence has implicated mitochondria as the end-effectors of IPC.

3.3. IPC and Mitochondria

The initial experimental studies to implicate mitochondria in the signaling pathway of IPC were designed to investigate the mechanism for the preserved myocardial ATP content in preconditioned hearts as originally proposed by Murry *et al* [1]. Over the years, the role of mitochondria in IPC has expanded them being potential trigger and effectors of cardioprotection. It appears that any stimulus which mildly stresses mitochondrial function is capable of initiating IPC. This mitochondrial stress then sets into place, through the activation of various pro-survival signaling pathways, a mitochondrial phenotype which is protected against a sustained lethal insult of IRI.

3.4. IPC and Myocardial Energy Production

In the initial IPC studies undertaken by Murry *et al* In 1990 [1, 65] first proposed that IPC may protect the heart by reducing myocardial energy demand during myocardial ischemia thereby preventing cell death by preserving myocardial ATP content and/or reducing catabolite accumulation. This proposal has been supported by a number of experimental studies reporting preserved mitochondrial function and maintained levels of high-energy phosphates [66]. The opening of the MitoKATP channel has been demonstrated to increase ATP synthesis [67], preserve mitochondrial energy production [68], decrease ATP hydrolysis [69] and improve energy transfer at reperfusion [69].

3.5. IPC and pH

During a sustained lethal episode of myocardial ischemia, the reduction in oxygen favors anaerobic glycolysis leading to ATP hydrolysis and the accumulation of lactic acid and a fall in the intracellular pH to 6.0. The increase in intracellular H^+ activates the Na^+-H^+ exchanger which extrudes H^+ ions in exchange for Na^+ ions resulting in intracellular Na^+ which in turn causes the Na^+-Ca^{2+} exchanger to work in reverse mode extruding Na^+ ions in exchange for Ca^{2+} ions the end result of which is intracellular Ca^{2+} overload. On reperfusing the ischemic myocardium the wash-out of lactate and the re-activation of ion pumps result in the rapid restoration of intracellular pH to pre-ischemic physiological levels of pH 7.4.

In 1990, Murry *et al* [65] first proposed that IPC may protect the heart by increasing glycogen depletion and reducing the rate of anerobic glycolysis during myocardial ischemia. Initial experimental studies did find glycogen depletion, decreased myocardial lactate, and less intracellular acidosis during myocardial ischemia in IPC-treated hearts [65, 70, 71], the expected result of which would be reduced activation of the Na^+-H^+ and Na^+-Ca^{2+} exchangers and less intracellular Ca^{2+} accumulation [72]. However, subsequent experimental studies have dissociated both glycogen depletion, decreased lactate accumulation and less intracellular acidosis with IPC cardioprotection [73, 74].

Interestingly, the rapid restoration of intracellular physiological pH at the onset of myocardial reperfusion did not appear to be affected by IPC [75], a finding which differs from IPost, which has been shown to delay the restoration of intracellular physiological pH within the heart, as a cardioprotective mechanism linked to inhibition of mPTP opening [76-78].

3.6. IPC and Calcium

Intracellular and mitochondrial calcium overload during myocardial ischemia and reperfusion can cause cardiomyocyte death by a number of different mechanisms including mPTP opening and cardiomyocyte hypercontracture [79, 80]. IPC has been reported to protect the heart by reducing intracellular calcium and mitochondrial calcium accumulation. The underlying mechanism for this beneficial effect of IPC on calcium handling is unclear, and has been attributed to opening of the sarcolemmal K_{ATP} channel (with action potential duration shortening) [81], opening of the MitoK_{ATP} channel (with partial mitochondrial membrane depolarization and less mitochondrial calcium accumulation) [82-90], and reduced intracellular acidosis during myocardial ischemia (see previous section) [72].

3.7. IPC and ROS

Reactive oxygen species (ROS) appear to play a dual role in the setting of IPC. On the one hand, the production of mitochondrial ROS (such as superoxide anion, hydrogen peroxide and hydroxyl radical) from the re-energization of the mitochondrial electron transport chain in the first few minutes of myocardial reperfusion mediates cardiomyocyte death by inducing mitochondrial permeability transition pore (mPTP) opening and causing cell membrane damage by lipid peroxidation. On the other hand the generation of small amounts of signaling ROS prior to the index episode of myocardial ischemia in response to a standard IPC stimulus is also required to mediate cardioprotection through the activation of pro-survival protein kinases such as PKC [91], Erk1/2 [42], and p38 MAPK [92].

3.7.1. IPC Attenuates Mitochondrial Production of ROS at Reperfusion

In 1994, Tosaki *et al* [28] first reported that IPC attenuated the production of ROS (detected indirectly by levels of malondialdehyde) at the onset of myocardial reperfusion in isolated perfused rat hearts. Using Lucigenin-enhanced chemiluminescence to directly measure levels of ROS in the isolated perfused rat heart, Crestanello *et al* [93, 94] found that IPC generated a burst of ROS immediately following the IPC stimulus and decreased the production of ROS in the first 4 min of myocardial reperfusion when compared to control hearts. These findings have been confirmed in a number of subsequent experimental studies [95-99]. However, the actual mechanism through which IPC attenuates mitochondrial production of ROS at the onset of myocardial reperfusion remains unclear. It has been suggested that IPC may prevent mitochondrial respiratory impairment during myocardial ischemia thereby resulting in less mitochondrial ROS production at time of myocardial reperfusion. Another hypothesis is that IPC somehow prevents the following process: during myocardial ischemia, outer mitochondrial membrane permeabilization allows the release of mitochondrial cytochrome C, the effect of which is increased mitochondrial ROS production [100].

Whether IPC can affect ROS production during myocardial ischemia is unclear. Experimental studies have demonstrated that minimal amounts of ROS were produced during myocardial ischemia and IPC had no effect on this [94, 95], whereas one experimental study has reported that IPC reduced the production of ROS in the last 10 min of myocardial ischemia in the isolated guinea pig heart [101]. In the latter study it was suggested that the ROS generated in the terminal phase of ischemia may somehow prime the heart for injury during myocardial reperfusion [101].

3.7.2. IPC Generates Signaling ROS Prior to Myocardial Ischemia

When present in low concentrations, reactive oxygen species (ROS), can modify cellular activities and participate in intracellular signalling [102]. In 1988, Murry *et al* [103] first demonstrated that antioxidants could abolish the protective effect of IPC, implicating for the first time a potential role for signalling ROS as a mediator of IPC, a finding which was later confirmed by several subsequent experimental studies [91, 104, 105]. In 1996, Crestanello *et al* [94] demonstrated that IPC generated a burst of ROS, a finding which has been confirmed in several studies [101, 106]. In 1997, Tritto *et al* [107] were the first to demonstrate directly that a low dose of ROS could mimic IPC-induced protection. A year later, Vanden Hoek *et al* [106] demonstrated using chick neonatal cardiomyocytes that the source of the preconditioning signaling ROS (mainly H_2O_2) was complex III of the mitochondrial electron transport chain. The mechanism through which IPC generates a burst of mitochondrial

signalling ROS has been attributed to the opening of the mitochondrial ATP-sensitive potassium (MitoK_{ATP}) channel (see the next section).

3.8. IPC and the Mitochondrial K_{ATP} Channel

The role of the K_{ATP} channel in IPC has had an interesting and often controversial history. In 1983, Noma [108] first identified a sarcolemmal ATP-sensitive potassium (K_{ATP}) channel in isolated guinea pig cardiomyocytes, the opening of which in response to hypoxia or ischemia could protect the heart against IRI by shortening the action potential duration thereby reducing intracellular Ca^{2+} loading. In 1992, Gross *et al* [109, 110] were the first to implicate the sarcolemmal K_{ATP} channel as a mediator of IPC, by demonstrating that IPC cardioprotection could be abolished by the sarcolemmal K_{ATP} channel blockers glibenclamide or 5-hydroxydecanoic acid (5-HD). Mice lacking the Kir6.2 component of the sarcolemmal K_{ATP} channel blockers have been reported to be resistant to IPC [111].

Following the discovery in 1991 of a K_{ATP} channel in the inner mitochondrial membrane [112], Garlid *et al* in 1997 [113] and Liu *et al* in 2008 [114] demonstrated a role for the MitoK_{ATP} channel as a trigger of IPC. The current paradigm suggests that the opening of the MitoK_{ATP} channel in response to the IPC stimulus is required to generate the mitochondrial signalling ROS required for the activation of downstream mediators of IPC cardioprotection such as PKC [115-119]. The mechanism through which MitoK_{ATP} channel activation generates ROS is unclear but the current proposition is that the opening of the mitochondrial K_{ATP} channel causes K^+ influx into the mitochondrial matrix coupled with H^+ efflux out of the mitochondria. The increase in matrix pH is accompanied by the influx of anions such as P_i, but because of the relatively low cytosolic concentration of P_i, the net effect of K^+ influx is matrix alkanization, which in turn increases mitochondrial ROS production from complex I [120-122].

However, the role of the MitoK_{ATP} channel as a trigger of IPC cardioprotection has been surrounded by controversy due to two main factors: (i) much of the evidence supporting the role of this channel in IPC is based upon the use of pharmacological activator and inhibitors of the MitoK_{ATP} channel such as diazoxide and 5-HD, respectively, agents which has been reported to have non-specific effects on mitochondrial function [123]; and (ii) although the molecular structure of the sarcolemmal K_{ATP} channel is known to consist of an octomeric complex containing four Kir6.2 (an inwardly-rectifying K^+ channel) subunits and four SUR2 (sulphonylurea receptor) subunits [124, 125], the molecular composition of the MitoK_{ATP} channel remains unknown. The situation has been further complicated with the discovery of a Ca^{2+}-activated K^+ channel in the inner mitochondrial membrane that can mediate protection against IRI [126].

3.9. IPC and Nitric Oxide

Whether the signalling molecule nitric oxide (NO) is a trigger for classical IPC was initially controversial [127-130]. However, in the current paradigm for IPC signaling, NO is a critical mediator in the IPC signalling pathways. The evidence for NO and iNOS in the setting of delayed IPC or SWOP is more persuasive [131, 132].

3.10. IPC and the mPTP

The mPTP is a non-selective channel of the inner mitochondrial membrane which forms and opens in the first few minutes of myocardial reperfusion in response to calcium, oxidative stress, phosphate and ADP [133]. Its opening collapses the mitochondrial membrane potential, halting mitochondrial oxidative phosphorylation resulting in ATP depletion and cell death. Furthermore, its opening allows water and solutes to equilibriate with the mitochondrial matrix leading to mitochondrial swelling and rupture of the outer mitochondrial membrane and the translocation of pro-apoptotic factors such as cytochrome C into the cytosol inducing apoptosis. Preventing its opening at the time of myocardial reperfusion is a critical target for cardioprotection. In this regard, Crompton's research laboratory made the crucial discovery that the opening of the mPTP induced by calcium, phosphate and oxidative stress could be pharmacologically inhibited by the immunosuppressant drug, cyclosporine-A (CsA), which targets cyclophilin D, regulatory component of the mPTP [134-136]. Since then we and others have demonstrated that pharmacologically inhibiting mPTP opening at the onset of reperfusion can reduce MI size [137-139], a therapeutic strategy which has been applied in the clinical setting [140]. Importantly, mice deficient in cyclophilin-D sustained smaller MI [135, 136] and cerebral infarcts [141], underscoring the importance of the mPTP as a mediator of IRI in both the heart and brain.

The first study to suggest that the mPTP may be a potential target for calcium-induced preconditioning protection was by Ashraf's group [142] in 2001, although no direct experimental evidence was provided to support this proposition. A year later, we postulated and demonstrated for the first time that IPC elicited its cardioprotective effect by targeting and inhibiting the opening of the mPTP [138]. We found that the preconditioning mimetic, diazoxide was able to reduce calcium-induced mPTP opening (measured by the loss of mitochondrial calcein) in adult rat cardiac mitochondria [138], suggesting a link between the MitoK_{ATP} channel and mPTP inhibition. Subsequent studies have confirmed mPTP inhibition in several different settings of preconditioning including: the preconditioned perfused rat heart [143, 144]; the preconditioned adult rat cardiomyocyte [145, 146]; the anesthetic-preconditioned *in vivo* rabbit heart [147]; and the rat heart protected by delayed preconditioning [148, 149]. The mechanism through which IPC actually inhibits the opening of the mPTP at the time of myocardial reperfusion is currently unresolved but several mechanisms have been proposed and are reviewed in a recent review [150].

More controversially, we have suggested that a transient non-lethal form of mPTP opening in response to the IPC protocol may contribute to IPC cardioprotection, by allowing the production of mitochondrial ROS to activate pro-survival kinases such as Akt [151, 152]. Recent imaging studies have reported flickering of the mPTP in intact cardiomyocytes under basal conditions associated with mitochondrial superoxide release [153].

4. SECOND WINDOW OF PROTECTION

One or more brief non-lethal episodes of myocardial ischemia and reperfusion can precondition the myocardium to withstand a sustained episode of lethal ischemia-reperfusion injury (termed classical or early ischemic preconditioning, IPC) [1]. The preconditioned state manifests immediately following the IPC stimulus and lasts for 1-2 hours after which it disappears [154]. In 1993, two research laboratories working independently of each other, made the intriguing discovery that the cardioprotective effect actually reappeared 24 hours later (termed the Second Window of Protection or delayed or late IPC). Yellon's group [155] observed infarct-size reduction using an *in vivo* rabbit model 24 hours following a standard IPC stimulus, suggesting and terming this the Second Window of Protection (SWOP). Kuzuya and co-workers [17] demonstrated myocardial infarct size reduction using an *in vivo* canine model either immediately or 24 hours following a standard IPC stimulus, but failed to observe an infarct-limiting effect at 3 or 12 hours following the IPC stimulus, confirming the presence of a bi-phasic cardioprotective response to IPC.

Delayed preconditioning can be elicited by both non-pharmacological stimuli (ischemia, heat stress, pacing and exercise) as well as pharmacological stimuli and can protect against myocardial infarction, myocardial stunning, arrhythmias and endothelial dysfunction. The studies which originally described delayed IPC used four-5 minute cycles of myocardial ischemia and reperfusion

to elicit delayed ischemic preconditioning (delayed IPC) in the rabbit heart [155] and canine heart [17], probably because this was the standard protocol used for eliciting early IPC. However, it has been shown that a single-5 minute cycle is also sufficient to elicit delayed IPC [156].

One of the first studies describing the phenomenon of delayed preconditioning had demonstrated that heat stress could limit myocardial infarct size 24 hours later and this beneficial cardioprotective effect was associated with increased myocardial levels of heat shock protein 72 KD (HSP72) [155]. However, the original experimental study to link heat stress with cardioprotection was published several years earlier by Currie and co-workers in 1988 [157], who first demonstrated that subjecting a rat to whole body hyperthermia (15 minutes at 42ºC) improved post-ischemic ventricular function, reduced cardiac enzyme release in excised perfused hearts, reduced ultra-structural damage to mitochondrial and decreased oxidative stress. Subsequent studies have linked heat stress induced delayed preconditioning with the generation of myocardial heat shock proteins and many of the mediators associated with delayed IPC.

4.1. Underlying Mechanisms

Since the original description of SWOP or delayed IPC 18 years ago, the mechanisms underlying this endogenous cardioprotective phenomenon have been the subject of intense investigation (see (Fig. **2**) for overview). Mechanistic pathways are needed in the SWOP (or delayed or late preconditioning) to relay the cardioprotective signal with respect to time, from the initial IPC stimulus to the cardioprotective effect which manifests 24 hours later. The signal transduction pathway underlying delayed IPC requires 'triggers' (substances generated during the IPC stimulus such as adenosine, bradykinin, opioids, cytokines, nitric oxide [NO] or reactive oxygen species [ROS]) which recruit 'early mediators' (such as PKC, tyrosine kinase, PI3K-Akt, MEK1/2-Erk1/2, and JAK), which in turn activate transcription factors (such as STAT1/3, NFκB, AP-1, Nrf2 and HIF-1α), resulting in the synthesis 12-24 hours later of 'distal mediators' (such as iNOS, HSP and COX-2) which protect the heart against infarction by acting on 'end-effectors' or 'targets' (such as the mPTP or the mK_{ATP} channel). The requirement for the transcription and synthesis of *de novo* protein cardioprotective mediators is one of the major factors distinguishing delayed from early IPC and it may account for the observed absence of cardioprotection in the 3-12 hours following the IPC stimulus. Clearly, this classification is not rigidly adhered to with some triggers/mediators/effectors difficult to classify under one heading as they may overlap one or more categories.

5. REMOTE ISCHEMIC CONDITIONING

"…preconditioning may be mediated by factor(s) activated, produced, or transported throughout the heart during brief ischemia/reperfusion." Przyklenk *et al* 1993 [8].

The disadvantages of IPC and also IPost are that they require an intervention being applied directly to the heart. Therefore, the ability to condition an organ or tissue away from the heart has facilitated the translation of RIC into the clinical setting. In 1993, Przyklenk and colleagues made the intriguing discovery that inducing brief episodes of ischemia and reperfusion in the circumflex coronary vascular territory had the capacity to reduce MI size, arising from the occlusion and reflow of the LAD coronary artery [8]. This form of intramyocardial protection was later extended to non-cardiac organs, with the report that MI size could actually be reduced in the animal heart by inducing brief ischemia and reperfusion in either the kidney [158] or the small intestine [159], immediately prior to the sustained coronary artery occlusion (reviewed in [160, 161]). The concept of RIPC has now been extended to different organs and tissues such that it has emerged as a true strategy of inter-organ protection against the detrimental effects of acute ischemia-reperfusion injury (IRI) (see (Fig. **3**) for overview). The discovery that RIPC could be elicited by inducing the remote ischemic conditioning (RIC) stimulus in the limb [162], by simply inflating and deflating a blood pressure cuff placed on the upper arm or thigh [13], has greatly facilitated the translation of RIC into the clinical setting.

5.1. The Remote Ischemic Conditioning Stimulus

The remote ischemic conditioning (RIC) stimulus is more closely related to the IPC protocols in that they are most often characterised by one to four cycles of brief ischemia and reperfusion (most often of 5-15 min duration). In the original study by Pryzklenk *et al* [8] four-5 min cycles of ischemia and reperfusion were applied to the heart. Interestingly, (most often of 5 min duration) whereas in the first demonstration of remote organ preconditioning by Gho *et al* [10], only one-15 min cycle of ischemia and reperfusion was applied to the remote organ (in this case intestine and kidney). The clinical translation of RIC required the stimulus to be applied to an organ or tissue which could be easily accessed. In this regard, the discovery that the skeletal muscle of the lower limb could be remotely conditioned to protect the heart against IRI [162] has facilitated the translational process with the subsequent demonstration that the remote conditioning stimulus could be applied to the upper or lower limb of human volunteers and patients to non-invasively protect the heart in clinical settings of IRI [13].

Interestingly, the RIC protocols have not varied much with respect to the timing of the intervention with respect to myocardial ischemia and reperfusion. That is similar protocols have been used in spite of whether it was delivered as a RIPC, RIPerC or RIPost protocol. In the first description of RIPerC by Schmidt *et al* in 2007 [11] four-5 min cycles of limb ischemia and reperfusion were applied after the onset of myocardial reperfusion. The term RIPost was first introduced by Kerendi *et al* in 2005 [163], although the one-5 min renal ischemia was applied during myocardial ischemia one minute prior to myocardial reperfusion. The first experimental study to actually begin the RIC protocol at the onset of myocardial reperfusion (the true definition of RIPost) was by Andreka *et al* in 2009 [12], who demonstrated that applying four 5 min cycles of limb ischemia and reperfusion, at the inset of myocardial reperfusion could reduce MI size using an *in situ* porcine model of IRI. The current paradigm of lethal myocardial reperfusion injury suggests that the cardioprotective intervention needs to be applied either prior to or at the immediate onset of myocardial reperfusion to be effective. However, in this RIPost study in which limb ischemia was initiated at the onset of myocardial reperfusion, the cardioprotective factor would have only reached the heart after the limb has been reperfused which would have been after 5 min of myocardial reperfusion had elapsed [12]. This intriguing finding suggests that there may be an additional component of lethal myocardial reperfusion injury which can be targeted, late into reperfusion.

5.2. Potential Mechanisms Underlying RIC

The actual mechanism through which an episode of brief ischemia and reperfusion in an organ or tissue exerts protection against a subsequent sustained insult of ischemia-reperfusion injury in a remote organ or tissue is currently unclear (see (Fig. **4**) for overview). Experimental studies suggest that many of the underlying mechanistic pathways and signal transduction cascades activated within the protected organ may be similar to those recruited in the setting of IPC and IPost [164]. Mechanistic pathways are needed to relay the cardioprotective signal from the remote preconditioned organ or tissue to the heart. However, once the cardioprotective signal has reached the heart, similar signaling pathways underlying IPC and IPost are then believed to mediate the protec-

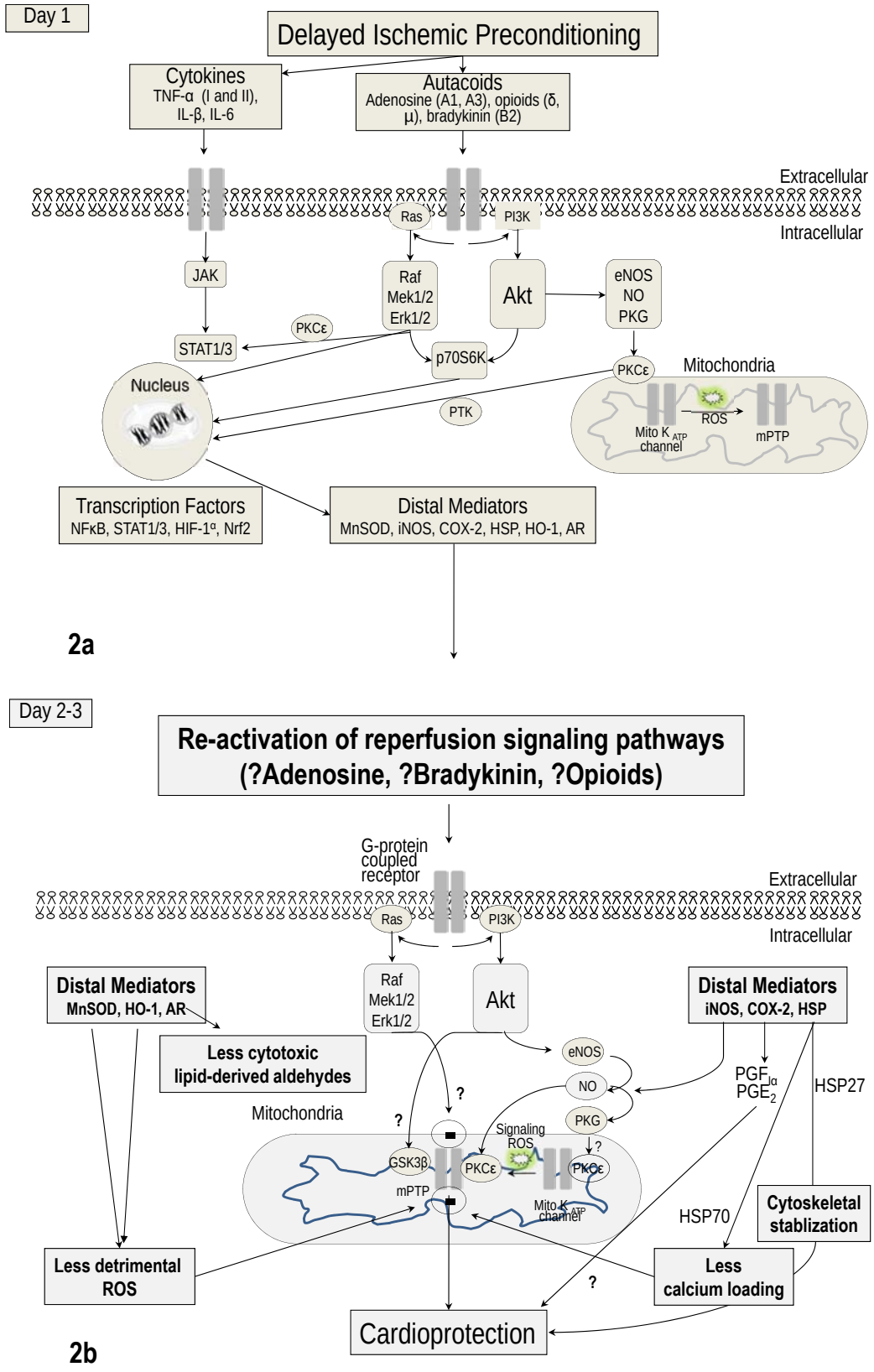

Fig. (2). Signaling pathways underlying delayed preconditioning or SWOP. Overview of major signal transduction pathways underlying delayed ischemic preconditioning (IPC) or the second window of protection (SWOP): (a) On day 1, delayed IPC generates trigger factors such as adenosine (acting via adenosine A1 and A3 receptors), opioids (acting via δ and μ opioid receptors) and bradykinin (acting via the bradykinin B2 receptor) which activate intracellular signalling pathways PIK3-Akt, Raf-MEK1/2-Erk1/2, and protein tyrosine kinase (PTK) which convey the cardioprotective signal to downstream pathways such as p70S6K, PKC-ε-STAT-3, eNOS-NO-PKG-PKCε-MitoK_{ATP}. This results in the nuclear activation of transcription factors such as NFκB, STAT1/3, HIF-1α,

Nrf2, which transcribe *de novo* proteins (distal mediators) over the next 12-24 hours such as MnSOD, iNOS, COX-2, heat shock proteins (HSP), heme-oxygenase-1 (HO-1), aldose reductase (AR), which then recruit cardioprotective pathways 24-48 hours later on the day of lethal insult of ischemia and reperfusion injury (IRI). In response to the delayed IPC stimulus the generation of cytokines such as TNF-α (acting via the TNF-α receptors I and II), IL-β, and IL-6, which activate the JAK-STAT pathway also resulting in the transcription of distal mediators. At the level of the mitochondria the opening of the mitoK_{ATP} channel results in the release of signalling reactive oxygen species, which further activate protein kinases such as Erk1/2 and Akt. The opening sensitivity of the mitochondrial permeability transition pore (mPTP) is decreased by the IPC stimulus, which confers cardioprotection 24-48 hours later in response to the IRI. (b) On day 2-3, the activated distal mediators confer cardioprotection at the time of myocardial infarction or stunning through a variety of pathways. The nitric oxide generated by iNOS mediates cardioprotection by inhibiting mPTP opening. The activation of COX-2 generates prostaglandins PGE_2 and $PGF_{1\alpha}$ which then mediate cardioprotection through an unclear mechanism. MnSOD and HO-1 exert an antioxidant effect reducing detrimental ROS generated during IRI which may mediate cardioprotection by inhibiting mPTP opening at the time of myocardial reperfusion. The activation of AR mediates cardioprotection by decreasing cytotoxic lipid-derived aldehydes. The pro-survival kinases such as Erk1/2 and Akt are activated at the onset of myocardial reperfusion and confer cardioprotection through mPTP inhibition via GSK3β and the mK_{ATP} channel. Whether autocoids such as adenosine, bradykinin or opioids activate these kinases at the time of myocardial reperfusion in unknown. The activation of HSP27 and HSP70 mediate cardioprotection by beneficial effects on stablization of the actin cytoskeleton and calcium regulation, respectively. (Figure reproduced from [18]).

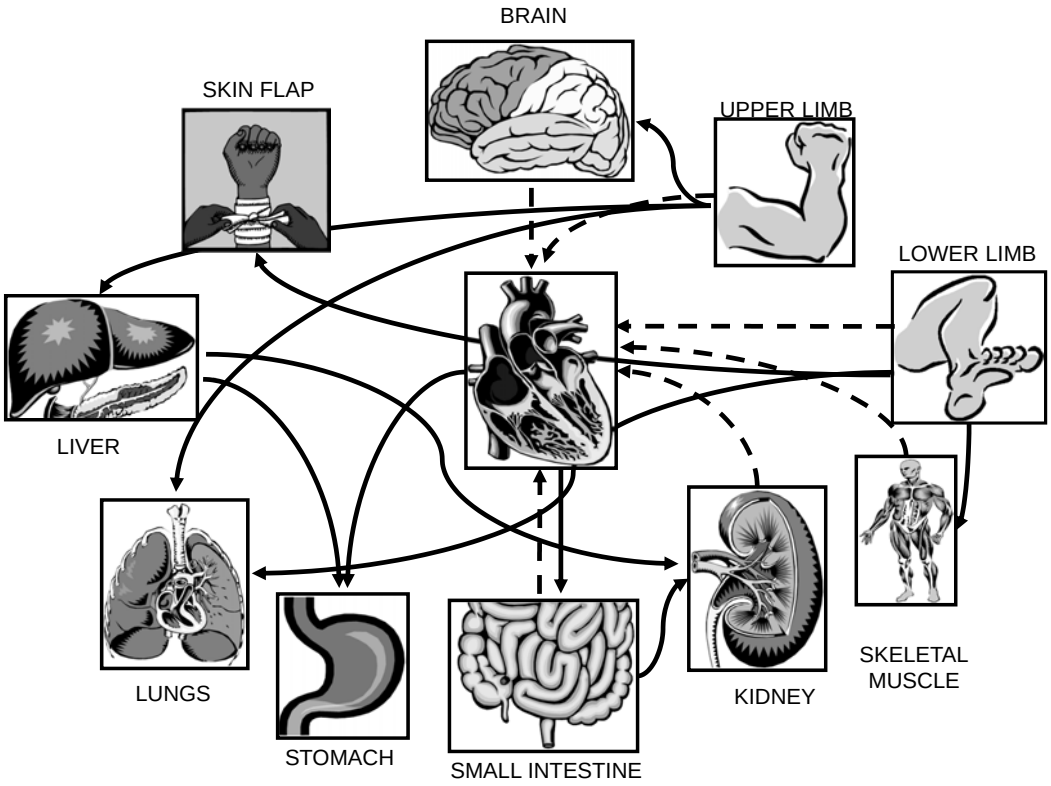

Fig. (3). Inter-organ protection against acute lethal ischemia-reperfusion injury. This cartoon depicts the evolution of remote ischemic preconditioning from a concept that was initially used to describe intramyocardial cardioprotection across coronary vascular territories. It was then demonstrated that brief ischemia and reperfusion of organs or tissue remote from the heart had the ability to protect the myocardium against acute lethal ischemia-reperfusion injury (see dashed black arrows). The concept has now been expanded between non-cardiac organs and tissues such that it represents a general form of inter-organ protection against acute lethal ischemia-reperfusion injury (solid black arrows link the remotely preconditioned organ or tissue to the protected target organ or tissue). (Figure reproduced from [7]).

tive effect. However, the mechanistic pathway linking the remote organ or tissue to the heart is currently unclear although several mechanisms have been proposed. It is important to appreciate that these mechanistic pathways may interact with each other and are therefore not mutually exclusive.

5.3. Evidence for a Potential Humoral Factor in RIC

The finding that sustained ischemia of the remote organ was unable to protect the heart and that a period of reperfusion of the remote organ was required, suggested that the reperfusion period may be needed to 'washout' a substance or humoral factor generated by the preconditioning ischemia, which was then transported to the heart [10, 158]. This hypothesis was further supported by a study reporting that blood taken from a rabbit which had been subjected to simultaneous IPC of both the heart and kidney, could reduce a subsequent myocardial infarct size by 77% when transfused to an untreated rabbit [165], suggesting the transfer of one or more humoral cardioprotective factors. The same authors went onto demonstrate that coronary effluent from an isolated rabbit heart treated with a standard ischemic preconditioning protocol, could reduce myocardial infarct size by 69% [166] and improve recovery of left ventricular function [167] when used to perfuse an untreated isolated rabbit heart.

Convincing evidence in support of a humoral mechanism for RIPC was provided in an elegant experimental study by Konstantinov *et al* [168]. Remote limb preconditioning of a pig that had received a donor heart was able to reduce myocardial infarct size in the denervated donor heart, providing strong evidence that a humoral mediator was responsible for RIPC protection, although an afferent sensory nerve pathway from the limb cannot be excluded [168]. A similar type of study was conducted by Kristiansen and colleagues [169] who demonstrated that hearts excised from a rat

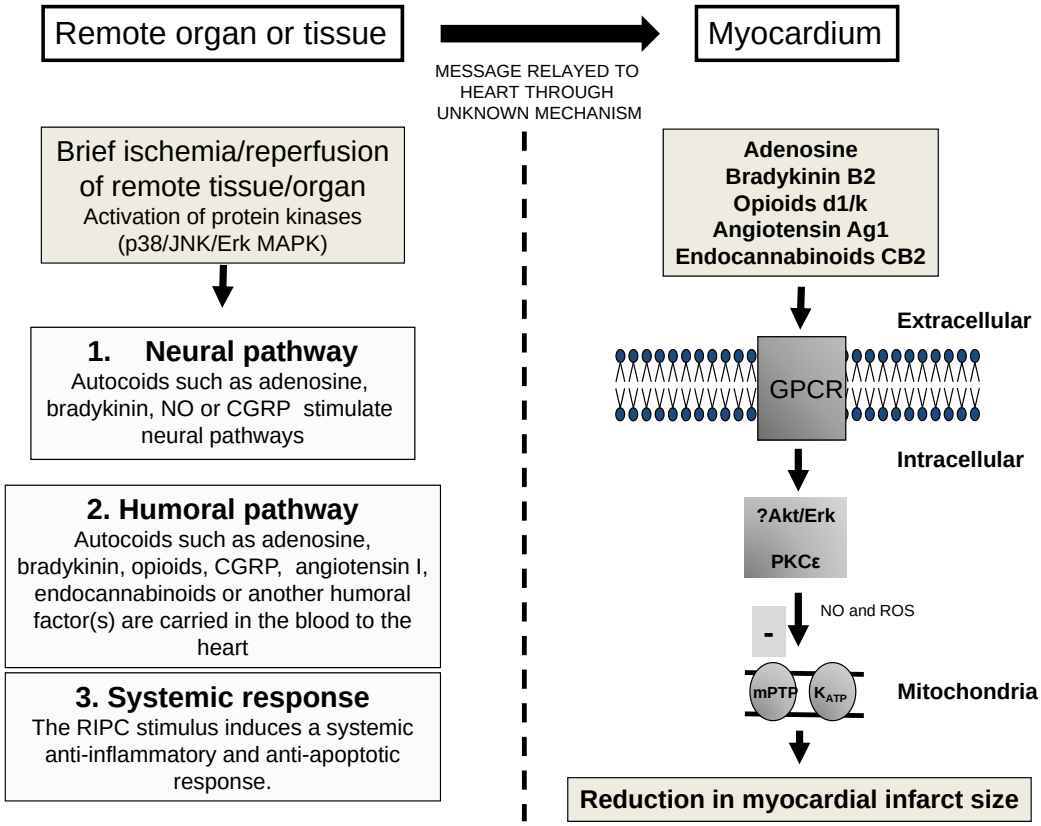

Fig. (4). Signaling pathways underlying remote ischemic preconditioning. The actual mechanism through which episode of brief ischemia and reperfusion in an organ or tissue remote from the heart protects the myocardium from acute IRI is currently unknown, although several hypotheses have been proposed and these are depicted in this figure: (1) The remotely preconditioned organ or tissue generates an endogenous substance such as adenosine, bradykinin or calcitonin gene-related peptide (CGRP), which then activates a local afferent neural pathway stimulating an efferent neural pathway, which terminates on the heart and mediates cardioprotection. (2) The remotely preconditioned organ or tissue generates an endogenous substance (such as adenosine, bradykinin, opioids, CGRP, endocannabinoids, Angiotensin I) or some other as yet unidentified humoral factor(s) which enters the blood stream and activates its respective receptor in the myocardium thereby recruiting the various intracellular pathways of cardioprotection; (3) The remotely preconditioned organ or tissue provokes a systemic protective response which suppresses inflammation and apoptosis. (Figure reproduced from [7]).

that had been remote limb preconditioned experienced a smaller infarct size, when subjected to IRI. Other studies have investigated whether endogenous substances such as adenosine [170], bradykinin [171], opioids [172], CGRP [173], and endocannabinoids [174], are released from the remote organ during the preconditioning ischemia and are carried to the heart in the blood stream where they then activate intracellular pathways of cardioprotection. Alternatively, the endogenous mediator may activate afferent neural pathways within the remote preconditioned organ to confer cardioprotection, as is the case with adenosine, bradykinin and CGRP.

In 1998, our laboratory was the first to implicate adenosine as a potential mediatory factor underlying cardioprotection in the setting of RIPC, demonstrating that the administration of the non-specific adenosine receptor antagonist, 8-sulphophenyltheophylline (8-SPT), prior to the RIPC protocol could abolish the reduction in myocardial infarct size induced by a remote preconditioning stimulus in the rabbit kidney [170]. In a subsequent study by Takao and colleagues [175], it was demonstrated that 8-SPT administered *after* the renal RIPC stimulus also had the ability to block cardioprotection suggesting that myocardial adenosine receptor binding was required for cardioprotection, a finding which was supported by their finding of elevated plasma levels of adenosine in blood sampled from the carotid artery of rabbits subjected to RIPC compared to those treated with IPC alone [175].

The involvement of opioid signaling in RIPC was first reported by Patel and colleagues in 2002 [172]. They demonstrated that the non-specific opioid receptor antagonist, naloxone, was capable of abolishing the myocardial infarct-limiting effects conferred by remote intestinal preconditioning in the rat [172]. It has been proposed that endogenous opioids generated by the preconditioning stimulus in the remote organ enter the blood stream where they act directly on the myocardium to confer cardioprotection [172], although further studies are required to both investigate this proposal and delineate the individual contributions of the different receptor subtypes to RIPC.

Previous studies have implicated binding at the CB2 endocannabinoid receptor of the endogenous cannabinoid system in protection from myocardial ischemia reperfusion injury [176]. A recent experimental study has implicated endogenous activation of the CB2 receptor in the myocardial infarct-limiting effects of remote intestinal preconditioning, using a pharmacological CB2 antagonist to abolish RIPC protection [174]. The authors proposed that endocannabinoids generated by the intestinal ischemia may enter the blood stream and activate CB2 receptors on the myocardium, but of course further studies are required to test this hypothesis.

5.4. What is the Identity of the Cardioprotective Factor in RIC

The possibility of a cardioprotective factor being transferred from the remote conditioned organ or tissue to the heart was first proposed in the initial RIC studies from 1993 [8] and 1996 [10], and yet the identity of the factor(s) involved remains unknown. Several experimental studies have attempted to identify the cardio-

protective factor(s) in remote preconditioned effluent or plasma using biochemical (chromatography) and mass spectrometry techniques but the identity of the protective factor(s) remains elusive [177-180]. To summarize, these studies have suggested that the cardioprotective factor(s) are probably thermo-labile hydrophobic substances (probably peptides) between 3.5 to 8 kDa in size which have been demonstrated to act via the opioid receptor, PKC, and the PI3K-Akt pathway [177-181]. Whether the same protective factor(s) is released by different remotely conditioned organs or tissues is unknown as is whether the factor(s) have similar protective effects depending on the target organ or tissue. Furthermore, the interplay between the cardioprotective factor(s) and the neural pathway remains unclear. Interestingly, it has been recently shown that the plasma from remote limb preconditioned human volunteers protected rabbit cardiomyocytes from simulated IRI, suggesting cross-species cardioprotection [180].

5.5. Evidence for a Potential Neural Pathway in RIC

One of the early studies of RIPC first provided potential evidence that a neural pathway may underlie the cardioprotection elicited by remote preconditioning a non-cardiac organ. Gho and colleagues [10] demonstrated that the reduction in myocardial infarct size induced by brief ischemia and reperfusion of the anterior mesenteric artery could be reversed in the presence of the ganglion blocker, hexamethonium. The hypothesis for a neural pathway was further developed with the proposition that endogenous substances such as adenosine [170], bradykinin [171], calcitonin gene-related peptide (CGRP) [173], released by the remote preconditioned organ, stimulated afferent nerve fibres, which then relayed to efferent nerve fibres terminating on the myocardium to confer cardioprotection.

Ding and colleagues [182] have demonstrated that renal nerve section abolished the cardioprotective effect induced by a remote renal preconditioning stimulus providing strong supportive evidence of a neural pathway. They then reported that during the remote renal preconditioning stimulus renal afferent nerve discharge was increased and that this enhanced neural activity could be abrogated by 8-SPT [182]. Further confirmatory evidence implicating adenosine in a neural pathway of cardioprotection was provided by Liem and colleagues [183] who after confirming that the prior administration of hexamethonium or 8-SPT abolished the myocardial infarct size reduction induced by brief mesenteric ischemia and reperfusion, demonstrated that the local administration of adenosine into the mesenteric vascular bed also conferred cardioprotection in a manner which was sensitive to hexamethonium [183]. These findings suggested that brief episodes of ischemia of the small intestine may generate adenosine which would then activate mesenteric afferent sensory nerves. However, the investigators went on to report that 8-SPT administered after the remote preconditioning stimulus was also able to inhibit cardioprotection, suggesting that adenosine receptor binding in the heart may also be required for protection [183].

Schoemaker & van Heijningen [171] demonstrated that the reduction in myocardial infarct size elicited by brief mesenteric artery occlusion and reperfusion could be abolished by prior administration of HOE140, a specific bradykinin B2 receptor antagonist. Interestingly, they went on to find that intra-mesenteric arterial administration of bradykinin was also able to confer cardioprotection in a manner which was sensitive to ganglion blockade by hexamethonium [171]. The authors suggested that bradykinin generated during the remote preconditioning intestinal ischemia, may stimulate mesenteric afferent sensory nerves which then mediate the cardioprotective effect [171]. These findings were confirmed in a subsequent study by Wolfrum and colleagues [184], who also observed that the activation of myocardial PKC-ε by brief intestinal ischemia was blocked by HOE-140 and hexamethonium, suggesting that PKC-ε was positioned downstream of bradykinin and the neural pathway.

Several experimental studies have implicated calcitonin-gene related peptide (CGRP), a neurotransmitter released from capsaicin-sensitive sensory nerves, as a potential mediator of both ischemic preconditioning [185] and RIPC [173, 186]. These can be summarized as follows: remote intestinal preconditioning generates nitric oxide which stimulates capsaicin-sensitive sensory nerves in the intestinal vasculature, releasing CGRP into the blood-stream (where levels have reported to be increased by RIPC), which is then carried to the heart where it activates myocardial PKC-ε. [185, 187].

5.6. Evidence for a Systemic Response in RIC

Several experimental studies have examined the effect of remote preconditioning of an organ or tissue on the myocardial gene transcription profile [188, 189], and the inflammatory response [190], and have discovered that the inflammatory response is suppressed and a favourable profile of gene transcription appears to be activated that is both anti-inflammatory and anti-apoptotic. The relevance of such a response to the cardioprotective effect elicited by RIPC is currently unclear and requires further investigation.

5.7. Myocardial Mechanisms of Cardioprotection in RIC

Once the cardioprotective signal has been conveyed from the remotely preconditioned organ to the heart, intracellular signal transduction mechanisms are recruited within cardiomyocytes which are similar to those that participate in IPC and IPost [191]. These include the ligand binding to G-protein cell surface coupled receptors such as adenosine [170], bradykinin [171], opioids [172], angiotensin [192], and endocannabinoids [174]. The binding to these cell surface receptors appears to then activate intracellular kinases such as PKC-ε [184] and other signalling components such as reactive oxygen species [193], nitric oxide and the mitochondrial KATP channel [170]. Whether RIPC also activates pro-survival kinases of the Reperfusion Injury Salvage Kinase (RISK) pathway and results in the inhibition of the mitochondrial permeability transition pore (mPTP), as in IPC and IPost, remains to be determined [164]. It has been recently shown that coronary effluent collected from IPC-treated hearts can protect naïve isolated perfused rat hearts when administered at the onset of myocardial reperfusion through the activation of the PI3K-Akt component of the RISK pathway [181].

5.8. Novel Concepts in RIC

Recent studies suggest that the heart can be protected from acute IRI by simply inducing surgical trauma elsewhere- a phenomenon which has been termed remote preconditioning of trauma (RPCT) [194, 195]. Jones *et al* [195] demonstrated that an abdominal surgical incision was enough to protect the murine heart from MI. The mechanistic pathway underlying this cardioprotective effect appeared to be mediated through a neural pathway involving sensory fibres, spinal nerves and cardiac sympathetic nerves which via bradykinin activated myocardial intracellular mediators of cardioprotection such as PKC and MitoKATP channel. Somewhat remarkably, the cardioprotection could be recapitulated by simply applying capsaicin cream to stimulate the C sensory fibres in the skin. RPCT has recently been confirmed in a canine model of IRI with a surgical abdominal incision reducing subsequent MI size [196].

A recent experimental study has suggested that the heart can be repeatedly remote ischemic postconditioned (RIPost) post-MI to elicit beneficial effects on LV remodelling [197]. Wei *et al* [197] found that repeating the RIPost stimulus either every day or every 3 days for 28 days reduced adverse LV remodelling post-MI and improved survival at 84 days post-MI. Whether repeated RIPost every 3 days, in post-MI patients has beneficial effects on LV remodeling is an intriguing possibility.

6. ISCHEMIC POSTCONDITIONING

In 2003, Zhao *et al* [19] made the surprising observation that interrupting myocardial reperfusion with several short-lived episodes of myocardial ischemia was cardioprotective. These authors found that by applying three-30 second cycles of alternating LAD reperfusion and LAD occlusion at the onset of myocardial reperfusion could reduce MI size by 44% in the canine heart [19]. Interestingly the term ischemic "postconditioning" had first been coined in an earlier experimental study by Na *et al* in 1996 [198] in which it was demonstrated that intermittent reperfusion achieved by ventricular ectopic beats could reduce reperfusion arrhythmias in a feline model of IRI. In fact, the concept of intermittent or gradual reperfusion as a cardioprotective strategy was first investigated in the 1980's [199, 200] but it is the term IPost which has captured the imagination of the research field of cardioprotection.

The discovery of IPost as therapeutic cardioprotective strategy which can be applied at the onset of myocardial reperfusion has revitalized interest in lethal myocardial reperfusion injury as a target for cardioprotection and provided solid evidence for the existence of lethal myocardial reperfusion injury in both animal models and man.

6.1. The IPost Stimulus

When compared to the IPC stimulus, which comprises one to four cycles of brief ischemia and reperfusion (most often of 5 min duration), the IPost stimulus is short-lived consisting of three to six cycles of ischemia and reperfusion (most often of 5 – 60 sec duration). In the original study by Zhao *et al* in 2003 [1], myocardial reperfusion was interrupted with three-30 sec cycles of myocardial ischemia. In IPC the brief episodes ischemia and reperfusion are required to induce a change in mitochondrial function whereas in IPost, it is probably the intermittent or stuttered reperfusion which is the most important aspect of the protocol. The IPost protocol varies from species to species with small animal myocardial models of IRI requiring 5 - 10 sec episodes of alternating ischemia and reperfusion whereas larger animal models and humans requiring 30 – 60 sec IPost protocols [201]. With respect to the timing of the administration of the IPost protocol, the current paradigm suggests that it needs to be delivered in the first minute of myocardial reperfusion to be effective. However, a recent experimental study has suggested in the murine model of *in situ* myocardial IRI, the IPost protocol may be still effective even when administered up to 45 min into myocardial reperfusion, suggesting that delayed postconditioning may be targeting a later component of myocardial reperfusion injury such as apoptosis or inflammation.

A number of pharmacological agents have been reported in experimental studies to reduce MI size when administered at the onset of myocardial reperfusion and have been termed pharmacological postconditioning agents despite the fact that many of them were investigated prior to the concept of IPost was introduced.

In addition to protecting the heart from the known proponents of lethal myocardial reperfusion injury such as oxidative stress, calcium overload, inflammation, mPTP opening, IPost is known to recruit a number of signal transduction pathways many of which are similar to those utilized by IPC.

6.2. Signaling Pathways Underlying IPost

The signal transduction pathways underlying IPost are both numerous and complex and only an overview can be given here. For more detailed accounts the reader is kindly directed to the following reviews [5, 202]. There are many similarities in the endogenous signal transduction pathways underlying classical or acute IPC and IPost in the cardiomyocyte. The obvious difference between the two, however, is the need to have a mechanism in place to mediate the 'memory' effect of IPC to sustain the cardioprotective effect for the first 2-3 hours. In contrast, the cardioprotective effect of IPost is manifested in the first few minutes of myocardial reperfusion. Whether the IPost stimulus also exerts remote protection to distant organs or tissues or whether it initiates a first and second window of protection in a similar manner to IPC is unknown. In common with IPC, the current paradigm is that the signaling pathway is initiated at the cell membrane surface with the activation of a variety of G-protein coupled receptors by a number of autacoids (such as adenosine, bradykinin, opioids), the activation of which recruit a wide variety of signal transduction pathways many of which appear to converge on the mitochondria.

6.2.1. Cell-surface Receptor Activation

The first G-protein coupled receptor (GPCR) to be linked to IPost was the adenosine receptor. An experimental study by Yang *et al* in 2005 [203] was the first to observe that the reduction in myocardial infarct size elicited by IPost could be abolished in the presence of 8-p-(sulfophenyl) theophylline (8-SPT), a non-specific adenosine receptor blocker. Interestingly, some 14 years earlier, the same research group had been responsible for first implicating adenosine receptor activation with the phenomenon of IPC [22]. The actual adenosine receptor subtype which is responsible for IPost is unclear with studies implicating the adenosine A1 [204], A2A [205], and A2B [206] receptor subtypes.

A subsequent study by Penna and colleagues [207] has implicated the endogenous activation of the GPCR, bradykinin B2, in IPost protection. These authors first demonstrated that two different pharmacological antagonists of the bradykinin B2 receptor abolished IPost protection in perfused rat hearts [207]. Finally, the recent finding that mice lacking the bradykinin B2 receptor were resistant to IPost protection, provides genetic evidence for the obligatory role of endogenous bradykinin B2 receptor activation in the setting of IPost [204]. The role for the bradykinin B1 receptor was less clear, as the mice were partially protected by the IPost stimulus [204].

Recently, Zatta and colleagues [208] have linked IPost with the endogenous activation of the opioid GPCR. After demonstrating that the non-specific opioid receptor antagonist, naloxone, was capable of abolishing IPost-protection in the intact rat heart, they investigated the effect of IPost in the presence of pharmacological antagonists of the δ-, κ-, and μ-opioid receptors [208]. The data implicated endogenous stimulation of the μ- and possibly the δ-opioid receptors in the setting of IPost [208]. In addition, hearts subjected to IPost accumulated higher levels of pro-encephalin, suggesting perhaps that IPost was capable of increasing endogenous opioid content in the reperfused myocardium [208]. Other receptors which have been implicated in IPost include the protease activated receptor 2 (PAR2) [209] and particulate guanylyl cyclase, the natriuretic peptide receptor [210].

6.2.2. Signal Transduction Pathways

A number of different signalling pathways have been investigated in the setting of IPost. The first of these was the Reperfusion Injury Salvage Kinase (RISK) pathway, a group of pro-survival protein kinases which on activation at the onset of myocardial reperfusion confer powerful cardioprotection [211, 212]. We and others have demonstrated that the pharmacological activation of components of the RISK pathway such as Akt and Erk1/2 at the immediate onset of myocardial reperfusion using a diverse variety of agents, which include growth factors, cytokines, GPCR agonists, natriuretic peptide, adipocytokines, and 'Statins' reduce myocardial infarct size in the region of 40-50% [211, 212]. Our laboratory and others have demonstrated that the cardioprotective benefits of IPost are dependent on the activation of Akt and Erk1/2 at the immediate onset of myocardial reperfusion [213, 214]. Subsequent studies have confirmed the role for Akt and Erk1/2 in the setting of IPost in both non-diseased animal hearts and diseased ones [215, 216] as well as human atrial muscle [217]. Interestingly, obese mice have been reported to be resistant to IPost protection, and this finding was associated with insufficient activation of the RISK pathway in

the hearts harvested from obese animals compared to control ones [218]. This finding underscores the importance of using relevant experimental animal models capable of simulating disease pathologies present in patients with coronary heart disease.

The Survival Activating Factor Enhancement (SAFE) pathway, which comprises the TNF-α receptor and Janus Kinase (JAK)-Signal transducer and activator of transcription (STAT) pathway, has also been linked to cardioprotection elicited by IPost [219, 220]. Experimental studies have reported that pharmacologically inhibiting the JAK-STAT pathway at the onset of myocardial reperfusion abrogates the infarct-limiting effects of IPost [219, 220]. However, mice with a cardiac-specific STAT3 deletion were found to still be amenable to the infarct-limiting effects of IPost, providing a suitable IPost protocol was used i.e. IPost using 5x5 sec cycles of ischemia/reperfusion reduced myocardial infarct size but 3x10 sec cycles did not [219]. Using mice with the same cardiac-restricted STAT-3 deletion, Goodman and colleagues [220] were able to demonstrate improved LV function using the IPost protocol of 3x10 sec cycles of ischemia/reperfusion, suggesting that STAT-3 may not be an obligatory mediator of IPost. The mechanism underlying the acute form of myocardial protection mediated by the JAK-STAT pathway is unclear but may relate to as yet unidentified mitochondrial effects [221].

Sphingosine kinase (SPK) is a lipid kinase which generates sphingosine 1 phosphate (S1P), which in turn regulates cell mitosis, apoptosis, cytoskeletal rearrangement, and survival [222]. Jin and colleagues [223] have recently demonstrated an obligatory role for SPhK1 as a mediator of IPost, which is potentially upstream of the RISK pathway. The authors reported that hearts isolated from mice lacking SPhK1, sustained larger myocardial infarcts, were resistant to IPost, and did not demonstrate activation of the Akt and Erk1/2 components of the RISK pathway in response to IPost [223].

It is well-established that protein kinase C acts as a critical mediator of protection in the setting of IPC, providing for the 'memory' elicited by an IPC-stimulus (reviewed in [224]). IPost has been reported to also be dependent on PKC activation. Penna and colleagues [225] were the first to demonstrate that the non-specific PKC inhibitor could abolish the infarct-limiting effects of IPost in perfused rat hearts, suggesting that IPost required the activation of PKC to confer cardioprotection. A subsequent study by Zatta and colleagues [226] has found that IPost-protection could be abolished by pharmacological inhibition of the PKC-ε isoform in early reperfusion. The translocation to the mitochondria of the detrimental PKC-δ, was reduced in postconditioned hearts. The mechanism through which IPost activates PKC is unclear. With respect to the cardioprotective effects of PKC, it has been postulated that PKC may sensitize the adenosine A2B receptor on the cell surface [206, 227] and that a special mitochondrial pool of PKC-ε confers inhibition of mitochondrial permeability transition pore (mPTP) opening [121].

Protein kinase G (PKG) has emerged as a critical mediator of cardioprotection in both IPC and IPost (reviewed in [228]). Much of the experimental data suggests that in the setting of IPC, it forms the final link in the signaling pathway which begins at the plasma membrane and terminates at the mitochondria [229]. In the context of IPost, its myocardial infarct-limiting effects have been demonstrated to be sensitive to pharmacological inhibition of the NO-sGC-cGMP-PKG pathway [203, 230]. Activated PKG at the level of the mitochondria is then believed to open the ATP-sensitive mitochondrial potassium channel through protein kinase C-ε [229]. This pathway is presumed to be activated through the Akt component of the RISK pathway via eNOS in response to IPost [213]. The downstream target of this pathway is believed to be PKC, resulting in sensitization of the adenosine A2B receptor [227] or the inhibition of mPTP opening [231, 232].

6.2.3. Mitochondria and IPost

Many of the signalling pathways underlying IPost appear to converge on the mitochondria. In this regard, the mitochondrial permeability transition pore (mPTP) has emerged as a critical target for cardioprotection in the setting of IPost (reviewed in [233, 234]). Preventing its opening at the onset of myocardial reperfusion using pharmacological mPTP inhibitors [138, 139, 233] or genetically ablating one of its critical components [135, 136, 235], reduces myocardial infarct size by 40-50%. IPost [236] has been reported to prevent mPTP opening at the onset of myocardial reperfusion, although the mechanism underlying this effect is currently unclear. It may involve components of the RISK pathway such as Akt, Erk1/2 or GSK-3β [237, 238] and/or changes in intracellular pH in the first few moments of myocardial reperfusion [76, 77]. Argaud and colleagues [236] found that mitochondria isolated from a perfused rabbit heart which had been subjected to a standard IPost protocol, were more resistant to calcium-induced opening of the mPTP, suggesting that IPost was capable of inhibiting mPTP opening. A subsequent study by the same group, reported that this inhibitory effect on mPTP opening was sensitive to pharmacological PI3K inhibition, suggesting that IPost mediated mPTP inhibition through the activation of the PI3K-Akt pathway [237]. We have demonstrated that mice lacking cyclophilin-D are resistant to IPost providing confirmatory evidence supporting the role of the mPTP in IPost [235]. Further studies suggest that the changes in cellular pH in early reperfusion of postconditioned hearts may also contribute to the inhibition of mPTP opening [77].

In the first few minutes of myocardial reperfusion there is a rapid restoration of physiological pH within the cardiomyocyte, mediated by the wash-out of lactic acid and the actions of the Na+-H+ exchanger (NHE) and the Na+-HCO- co-transporter. Interestingly, IPost [76, 77, 239] has been reported to delay the restoration of physiological pH in the early moments of myocardial reperfusion. This transient acidosis in the first couple of minutes of reperfusion may be sufficient to permit the activation of the RISK pathway [76], suppress mPTP opening [77], inhibit cardiomyocyte hypercontracture, and prevent detrimental calpain activation [239], over this critical period of time. The mechanism through which IPost modifies cellular pH in early reperfusion is currently unclear, but it has been attributed to delayed wash-out of lactate, but another potential explanation could be the inhibition of the NHE by the RISK pathway, which is activated in IPost-treated hearts. A recent study by Avkiran's laboratory [240] has demonstrated that Akt is able to phosphorylate and inhibit the actions of NHE in cardiomyocytes, and so whether this process occurs in postconditioned hearts is an interesting possibility.

Previous experimental studies have suggested that the ATP-sensitive mitochondrial potassium (MitoKATP) channel plays a pivotal role in IPost, although much of the evidence is circumstantial and based on the use of pharmacological activators and inhibitors of the channel. Studies have demonstrated that the pharmacological inhibition of the MitoKATP channel in early reperfusion abolished the infarct-limiting effects of IPost [214, 241, 242].

IPost [19] has been reported to reduce the amount of detrimental ROS generated at the onset of myocardial reperfusion. In contrast, ROS may also participate as critical signaling mediators in the signal transduction pathway underlying IPost [225]. Penna and colleagues [225] were the first to demonstrate that the administration of the non-specific ROS scavenger, N-acetylcysteine (NAC), was able to abrogate the infarct-limiting effect of IPost in perfused rat hearts. Crucially, if NAC was administered after the first three minutes of myocardial reperfusion had elapsed, no effect on IPost was observed, suggesting that ROS signaling during the first couple of minutes of myocardial reperfusion was critical to IPost protection [225]. Subsequent studies using intact rabbit [243] and murine [244] hearts have confirmed the involvement of a signaling form of ROS in IPost protection.

7. EMERGING CONCEPTS IN ISCHEMIC CONDITIONING

7.1. Combining Different Ischemic Conditioning Stimuli

The difference in the 'timing' and the 'site or delivery' of the 3 different forms of ischemic conditioning, provide the possibility of combining different ischemic conditioning protocols to achieve either additive or synergistic cardioprotection. The belief that underlying mechanisms may be similar across the three forms of ischemic conditioning and the limitations of the current animal models of IRI in terms of resolution of cardioprotection may also have contributed to the lack of enthusiasm for combining different protocols. IPC has been tried in combination with IPost by several investigators with inconclusive findings [213, 245, 246]. Recent experimental studies have combined RIPerC with IPost protocols and observed additive cardioprotection, although no additive effect was observed when combined with IPC [247]. Further investigation is now required to explore the possibility of combining ischemic conditioning protocols in the clinical setting.

7.2. Gene Therapy for Cardioprotection

The identification of iNOS as a distal mediator of delayed cardioprotection has been investigated as a therapeutic strategy using gene therapy to adenovirally transfect adult murine hearts with iNOS, a strategy which resulted in infarct-size limitation, through a mechanism involving COX-2 [248]. Similar findings have been reported with Ec-SOD adenoviral transfection [249, 250]. The same authors are exploring the possibility of adenoviral transfection of other known distal mediators (such as COX-2) of delayed cardioprotection as a strategy for long-term 'prophylactic cardioprotection' in animal models (reviewed in [251]). The possibility of maintaining the heart in a state of cardioprotection is particularly attractive given the unpredictable onset of acute coronary syndromes. However, the long-term consequences of prolonged activation of cardioprotective mediators such as iNOS and COX-2 would have to be investigated. In this regard, Li *et al* [252, 253] have demonstrated that adenoviral transfection of murine hearts with heme oxygenase-1 or iNOS resulted in long-term cardioprotection with MI size reduction at one year with no adverse functional effects.

7.3. Regenerative Therapy for Cardioprotection

Regenerative therapy using stem cells as a treatment strategy for coronary heart disease remains an ongoing area of research interest and has been recently been investigated in the setting IPC in two areas. In the first area, IPC has been used as a therapeutic strategy for improving survival and engraftment of bone marrow derived stem cells [254]. The second area the focus has been on the potential role of bone marrow derived stem cells as 'mediators' of delayed IPC. Li and co-workers [255] found using an *in vivo* murine model that a standard IPC stimulus could recruit into the myocardium bone marrow-derived endothelial progenitor cells (EPC) within 3 hours, and that these cells 'imported' cardioprotective mediators such as iNOS and eNOS into the ischemic myocardium. In human volunteers, repeated episodes over one month of remote ischemic preconditioning induced by brief cycles of ischemia and reperfusion of the arm, has been reported to be associated with an increase in circulating EPC's and improved endothelial function [256], but the contribution of the EPC's to these beneficial endothelial effects are unclear. In a subsequent study, Kamota and co-workers [257] demonstrated using an *in vivo* murine model that IPC induced by brief cycles of occlusion and reperfusion of the aorta (remote preconditioning stimulus) had several beneficial effects 24 hours later including reduced myocardial infarct size, preserved LV ejection fraction, augmented peripheral circulating levels of CD34+ and flk-1+ bone marrow stem cells (BMSC), increased myocardial targeting to the ischemic risk zone of GFP-tagged BMSC. The IPC stimulus was reported to increase levels of serum vascular endothelial growth factor and stromal cell-derived factor-1α [257]. Crucially, using antibodies to abrogate the IPC-induced elevation and targeting of BMSC, also abolished the cardioprotective benefit. Clearly, the mechanism underlying the cardioprotective benefits of BMSC are unclear but over a relatively short time interval (24 hours) [257], the release of cardioprotective humoral factors by BMSC into the infarcted myocardium would seem a possible explanation.

7.4. Neural Pathways in Cardioprotection

It has been suggested that neural pathways are involved in mediating the cardioprotective stimulus from the remote conditioned organ or tissue to the target organ or tissue in the setting of RIC [7]. Whether neural pathways are also involved in IPC is an interesting unanswered question. Kudej and co-workers [258] demonstrated that regional cardiac denervation of the porcine heart abrogated the *in vivo* infarct-limitation provided by delayed IPC and prevented the upregulation of myocardial iNOS and COX-2, established mediators of delayed cardioprotection. However, the cardioprotection elicited by early IPC was not affected [258]. Whether cardiac innervation is required during the IPC stimulus or at the time of myocardial infarction is unclear. Clearly, further work needs to be undertaken to examine the role cardiac nerves contribute to delayed IPC. In this respect, hearts excised from remote limb preconditioned rats generated smaller infarcts on the Langendorff-apparatus [169], suggesting that cardiac innervation at the time of infarction may not be necessary for cardioprotection, presumably because the heart was already in a preconditioned state.

7.5. MicroRNAs and Cardioprotection

MicroRNAs (miRNAs) are noncoding RNAs involved in the post-transcriptional regulation of protein expression, which have been recently implicated in cardiac development, cardiac hypertrophy, cardiac failure and arrhythmogenesis (reviewed in [259]). It has been demonstrated that miRNA's may contribute to classical IPC cardioprotection [260]. Yin and co-workers [261] initially demonstrated that heat stress of mice increased peripheral circulating levels of miRNA's 1, 21, and 24, which when isolated and injected into naïve mice conferred cardioprotection 24 hours later in Langendorff-perfused hearts. A subsequent study by the same groups found a similar serum miRNA profile produced in response to a standard IPC [262]. In this case, the isolated and purified miRNA were injected into the LV wall and resulting in infarct size limitation 48 hours later, a finding which was associated with upregulation of eNOS, HSP70, and the HSP70 transcription factor (HSF-1) [262], known mediators of delayed cardioprotection. Clearly, further work is required to determine how the IPC stimulus generates miRNA and which proteins or kinases are modified by miRNA in the context of delayed IPC. Another intriguing possibility is whether miRNA could act as the humoral factor(s) conveying cardioprotection from the distal remote preconditioned organ or tissue to the heart.

8. CONCLUSION

The phenomenon of ischemic preconditioning has evolved into a number of different forms including remote ischemic conditioning and ischemic postconditioning, a process which has facilitated its translation into the clinical setting of cardioprotection. Ongoing experimental studies continue to elucidate the mechanisms underlying these 3 different forms of endogenous cardioprotection and over the years huge progress has been made towards the understanding of adaptation of the cardiomyocyte to ischemia-reperfusion injury and the intracellular signal transduction pathways that mediate this process. Proof-of-concept clinical studies have demonstrated beneficial effects with ischemic preconditioning, ischemic postconditioning and remote ischemic conditioning in a variety of clinical settings and reviewed in another article in this special issue. Therefore, harnessing the powerful cardioprotection elicited by ischemic preconditioning, remote ischemic conditioning, and ischemic postconditioning could one day provide a therapeutic strategy for pro-

tecting the heart against acute IRI, preserving cardiac function and improving clinical outcomes in patients with ischemic heart disease.

CONFLICT OF INTEREST

The authors confirm that they have no conflicts of interest.

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