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Themed Section: Conditioning the Heart – Pathways to Translation

REVIEW

Signalling pathways and mechanisms of protection in pre- and postconditioning: historical perspective and lessons for the future

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Ischaemic pre- and postconditioning are potent cardioprotective interventions that spare ischaemic myocardium and decrease infarct size after periods of myocardial ischaemia/reperfusion. They are dependent on complex signalling pathways involving ligands released from ischaemic myocardium, G-protein-linked receptors, membrane growth factor receptors, phospholipids, signalling kinases, NO, PKC and PKG, mitochondrial ATP-sensitive potassium channels, reactive oxygen species, TNF-α and sphingosine-1-phosphate. The final effector is probably the mitochondrial permeability transition pore and the signalling produces protection by preventing pore formation. Many investigators have worked to produce a roadmap of this signalling with the hope that it would reveal where one could intervene to therapeutically protect patients with acute myocardial infarction whose hearts are being reperfused. However, attempts to date to show efficacy of such an intervention in large clinical trials have been unsuccessful. Reasons for this inability to translate successes in the experimental laboratory to the clinical arena are evaluated in this review. It is suggested that all patients with acute coronary syndromes currently presenting to the hospital and being treated with platelet $P2Y_{12}$ receptor antagonists, the current standard of care, are indeed already benefiting from protection from the conditioning pathways outlined earlier. If that proves to be the case, then future attempts to further decrease infarction will have to rely on interventions which protect by a different mechanism.

LINKED ARTICLES

This article is part of a themed section on Conditioning the Heart – Pathways to Translation. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-8

Abbreviations

AMI, acute myocardial infarction; Cx43, connexin 43; GP, glycoprotein; GSK-3β, glycogen synthase kinase-3β; HB-EGF, heparin-binding EGF-like growth factor; IPC, ischaemic preconditioning; IPoC, ischaemic postconditioning; K_{ATP}, ATP-sensitive K^+ channel; mPTP, mitochondrial permeability transition pore; mtK_{ATP}, mitochondrial K_{ATP}; NHE, Na^+/H^+ exchanger; PAF, platelet-activating factor; PCI, percutaneous coronary intervention; PDK, 3′-phosphoinositide-dependent kinase; PTCA, percutaneous transluminal coronary angioplasty; RISK, reperfusion injury survival kinases; ROS, reactive oxygen species; S1P, sphingosine-1-phosphate; SAFE, survivor activating factor enhancement; SPHK1, sphingosine kinase; Src, sarcoma; STEMI, ST-segment-elevation myocardial infarction; TRAF-2, TNF receptor-associated factor 2

Tables of Links

TARGETS	
GPCRs[a]	**Transporters**[d]
β-adrenoceptor	Na^+/Ca^{2+} exchangers
A_1 receptor	Na^+/H^+ exchangers (NHE)
A_{2A} receptor	**Enzymes**[e]
A_{2B} receptor	Akt
A_3 receptor	COX
$S1P_1$ receptor	eNOS
$S1P_2$ receptor	ERK
$S1P_3$ receptor	GSK-3β
Ion channels[b]	Guanylyl cyclase
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Catalytic receptors[c]	PKCε
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Desflurane	Tirofiban
Erythropoietin	Tyrosine
Exenatide	Urocortin
Glibenclamide	Wortmannin
Insulin	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b,c,d,e]Alexander *et al.*, 2013a,b,c,d,e).

Introduction

With the exception of revascularization, ischaemic preconditioning (IPC) is undeniably the most powerful cardioprotective intervention targeting ischaemia/reperfusion injury yet to be identified. All scientists agree that this intervention can salvage ischaemic myocardium following a period of ischaemia and reperfusion and reduce infarct size, the original observation and time-honored parameter of cardioprotection. Yet this success in the experimental laboratory has yet to be translated into a clinical procedure that has produced equally satisfying results. Thus, both scientists and clinicians continue to search for the miraculous intervention that can be applied to the patient with an acute myocardial infarction (AMI) to decrease infarct size and diminish the clinical sequelae of coronary artery occlusion and reperfusion. The closest we have come to this is revascularization therapy. In patients with coronary occlusion and AMI, current standards demand that the coronary artery be opened to reperfuse the ischaemic myocardium. Although tissue salvage understandably is dependent on reflow, this revascularization paradoxically creates injury of its own, so-called 'reperfusion injury'. It is the latter which most recent cardioprotective interventions purport to target. To appreciate why identification of an appropriate cardioprotective agent has largely failed to date and to provide hope that we may be close to developing an effective approach, it is necessary to understand the history and science of cardioprotection.

Pioneering studies of infarct size modification

ST-segment shifts as a marker of infarct size

In 1971, Maroko, working in Braunwald's laboratory, proposed strategies for limiting necrosis following acute coronary occlusion (Maroko *et al.*, 1971). Maroko *et al.* recognized the importance of infarct size on outcomes following infarction and were the first to suggest that infarction might be therapeutically reduced. These investigators mapped the degree of ST-segment shifts on the anterior myocardial surface at the end of 10-min coronary occlusions in dogs. After the heart was reperfused and had recovered, the occlusion and mapping were repeated after an experimental intervention. The sum of the ST-segment shifts was thought to reflect the severity of the ischaemia. If the sum of ST-segment shifts was attenuated, it was concluded that the intervention had protected the heart. The concept was brilliant in that it allowed each animal to serve as its own control, but it relied on the assumption that the ST-segment sum represented true infarct size which, unfortunately, turned out to not always be the case. Maroko *et al.* as well as subsequent investigators

were also greatly handicapped by a lack of scientific information as to how ischaemia/reperfusion actually kills heart tissue. The Braunwald laboratory focused on a supply–demand relationship. Demand could be reduced by β-blockers and supply could be increased by interventions thought to promote oxygen delivery such as hyaluronidase (Maroko *et al.*, 1972). As it turned out, the supply–demand relationship was but only one determinant of cell death. Yet their pioneering efforts started a field of research that still thrives today.

Reperfusion injury: a paradox

Hearse *et al.* (1973) introduced the 'oxygen paradox'. Perfusion of a rat heart with hypoxic buffer for a prolonged period seemed to have little consequence, but switching back to oxygenated perfusate caused immediate cell destruction. While the reintroduction of oxygen was needed for recovery, at the same time it was associated with an injury. That was the paradox. The concept of reperfusion injury was very attractive because at that time it was recognized that AMI was caused by a coronary thrombus that could be dissolved with a thrombolytic agent. If much of the injury occurred at reperfusion, it would not be too late to prevent it with some intervention despite presentation of the patients with ischaemia in progress. It was hypothesized that reintroduction of oxygen produced a burst of free radicals that in turn led to membrane damage, interference with ion pumps and volume dysregulation. A closely associated hypothesis was that leukocytes would invade reperfused tissue and attack viable myocytes by releasing free radicals. Personnel in Lucchesi's laboratory concentrated on the role of free radicals in myocardial infarction (Jolly *et al.*, 1984). Thus, free radical scavengers appeared to decrease infarction in a canine model of ischaemia/reperfusion. Although these studies were championed by local advocates, the inconsistent results obtained in other independent laboratories suggested problems with this approach (Reimer *et al.*, 1989). The same held for investigations of anti-inflammatory agents (Tissier *et al.*, 2007a). The reason for divergent results among the many studies of antioxidant and anti-inflammatory agents have never been resolved, but even in the most supportive studies salvage was hardly greater than 10%, probably too modest to have meaningful clinical impact. One began to wonder if it was even possible to alter the vulnerability of ischaemic myocardium to infarction.

IPC

Then, in 1986, Charles Murry, in the laboratory of Reimer and Jennings, made a seminal observation (Murry *et al.*, 1986). It was reported that preceding a 40 min coronary occlusion in dogs with four cycles of 5 min coronary occlusion/5 min reperfusion would decrease the amount of infarction of the risk area subtended by the occluded vessel from 28 to 7%. That was a 75% reduction in infarct size despite the fact that those hearts endured an additional 20 min of ischaemia. They called this phenomenon IPC. Perhaps because of Murry's frankly antithetical observation that more ischaemia was better, confirmation of the observation was not immediate. Three years passed before scientific papers dealing with IPC began to appear. But when they did, confirmation was overwhelming. Those studies noted that the intervention uniformly protected canine (Murry *et al.*, 1986; Gross and Auchampach, 1992), rodent (Liu and Downey, 1992; Yellon *et al.*, 1992), porcine (Schott *et al.*, 1990), rabbit (Van Winkle *et al.*, 1991; Toombs *et al.*, 1993), primate (Yang *et al.*, 2010) and even avian (Rischard and McKean, 1998) hearts from myocardial infarction. At last there was conclusive proof that infarct size could be modified, at least by this singular intervention of IPC. Of course, therapeutic IPC of a heart would be impossible to implement clinically in any setting except, perhaps, open heart surgery. Translation of IPC into clinical practice would have to wait until its mechanism was better understood before a treatment could be identified that could be administered after ischaemia had begun.

Mechanism of IPC: triggering phase

Surface receptors trigger IPC

The first insight into IPC's mechanism was reported by Liu *et al.* (1991). They announced that IPC is triggered by receptor occupancy. Activation of the G_i-coupled adenosine A_1 receptor in rabbits triggered IPC's protection. Thus, an adenosine receptor antagonist blocked IPC's protection, while infusion of adenosine or an A_1-selective agonist in lieu of brief ischaemia duplicated IPC's protection. Liu *et al.* proposed that net dephosphorylation of ATP during ischaemia results in production and release of adenosine which then would bind to A_1 adenosine receptors leading to a preconditioned phenotype. So had these investigators defined IPC's mechanism and were they ready to propose an intervention that could be used clinically? Hardly. They had identified a pharmacological trigger, but unfortunately the trigger, like IPC, had to be given prior to the onset of ischaemia. Identification of more parts of IPC's signal transduction pathway and of the overall mechanism would be required.

Opioid and bradykinin's signalling

Two other endogenously released trigger substances, bradykinin (Wall *et al.*, 1994) and opioids (Schultz *et al.*, 1995), were also found to be involved in IPC's protective action. Inhibition of any of these three receptors aborted protection from a single preconditioning cycle. However, simply increasing the number of preconditioning cycles could restore protection suggesting that the three receptors had an additive effect which was required to reach a protective threshold (Goto *et al.*, 1995). Thus, the additional cycles of ischaemia/reperfusion produced increased stimulation of the two uninhibited receptors so that the protective threshold could finally be reached.

All three of these triggers, adenosine, bradykinin and opioids, bind to G_i-coupled receptors. The proposed multiple trigger theory implies that all triggers converge on a common target. Ytrehus *et al.* (1994) reported that PKC seemed to play a major role in IPC and it was found that protection triggered by any of the three receptors could be blocked by PKC inhibitors (Goto *et al.*, 1995; Sakamoto *et al.*, 1995; Baines *et al.*, 1997; Miki *et al.*, 1998a). Thus, PKC is believed to be this common target. Hence, adenosine, bradykinin and opioids bind to their respective receptors and the second messenger

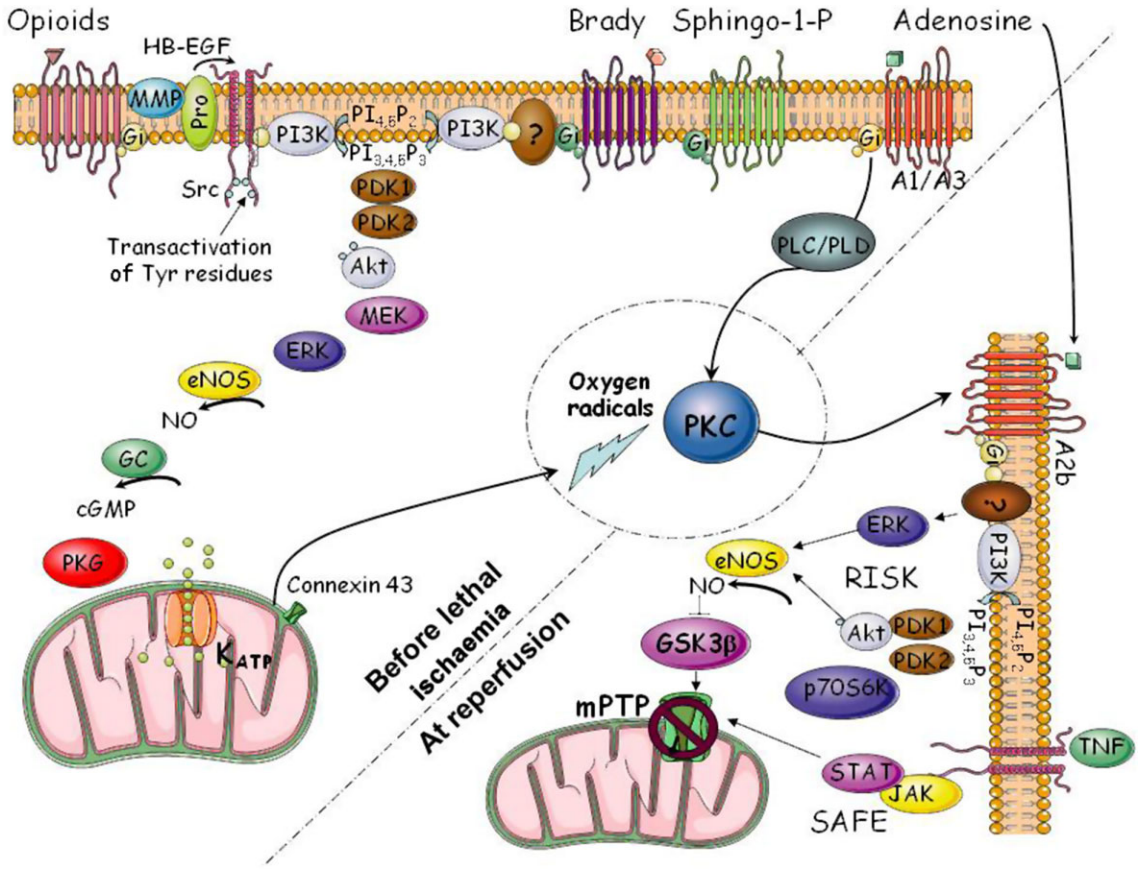

Figure 1

Proposed signalling scheme for conditioning. Abbreviations: Brady, bradykinin; eNOS, endothelial NOS; K_{ATP}, ATP-dependent potassium channel; MEK, MAPK kinase; MMP, matrix metalloproteinase; p70S6K, p70S6 kinase; PDK1/2, 3′-phosphoinositide-dependent kinase-1/-2; $PI_{3,4,5}P_3$, phosphatidylinositol trisphosphate; $PI_{4,5}P_2$, phosphatidylinositol bisphosphate; Pro, pro-HB-EGF; Sphingo 1-P, sphingosine 1-phosphate; Src, sarcoma tyrosine kinase; TNF, TNF-α; Tyr, tyrosine. Modified from Tissier *et al.* (2007a).

G-protein is cleaved into active α and βγ subunits which then result in activation of PKC. It would seem intuitive that the various agonists coupled to common G_i-proteins should trigger identical signalling. However, mysteriously this is not the case. Adenosine, bradykinin and opioids activate very divergent pathways; however, all three pathways eventually converge on PKC.

Opioid cardioprotection is dependent on downstream metalloproteinase and EGF receptor (Cohen *et al.*, 2007a). This part of the signalling pathway was first mapped by studying ACh-stimulated receptors, G_i-coupled receptors, whose downstream protection is governed by signalling similar to that of opioids (Krieg *et al.*, 2002; 2004; Oldenburg *et al.*, 2002; 2003). While ACh is a potent trigger of preconditioning's protection, it is not a physiological trigger as transient ischaemia does not cause its release. ACh binds to its receptor resulting in cleavage of G_i and subsequent metalloproteinase-dependent cleavage of heparin-binding EGF-like growth factor (HB-EGF) from membrane-associated pro-HB-EGF (Figure 1). The liberated HB-EGF then activates membrane-bound EGFR by binding to its ectodomain resulting in EGFR dimerization which in turn leads to autophosphorylation of tyrosine residues on both EGFRs and binding of sarcoma (src) tyrosine kinase to form a signalling module. The latter attracts and activates PI3K.

Bradykinin's signalling is comparable, although a different metalloproteinase is involved. Methner *et al.* (2009) demonstrated involvement of metalloproteinase-8 and the EGFR. Downstream steps are similar to those for ACh and opioids (Cohen *et al.*, 2007a).

NO

PI3K-produced phosphorylated lipid metabolites phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate induce Akt to translocate to the plasma membrane (Andjelković *et al.*, 1997) where it is phosphorylated by PDK1 and 2 (Stephens *et al.*, 1998) and this initiates a signalling cascade. Akt activates ERK and endothelial NOS (Dimmeler *et al.*, 1999). The latter enzyme catalyzes production of NO which stimulates guanylyl cyclase (GC). GC catalyzes the production of cGMP which itself activates PKG (Figure 1).

NO is a gaseous free radical and important biological regulator and cellular signalling molecule. In 1992, Vegh *et al.* (1992) proposed that endogenous NO might be involved in preconditioning. This announcement triggered a significant

controversy regarding the precise role of NO in IPC (Weselcouch *et al.*, 1995) to which we unwittingly contributed. In a study in *in vitro* rabbit hearts published in 2000, we noted that N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME), a NOS inhibitor, had no effect on the dramatic protection induced by IPC, whereas the NO donor S-nitroso-N-acetylpenicillamine administered before the index ischaemia in lieu of the repeated brief 5 min coronary occlusions mimicked IPC and protected hearts (Nakano *et al.*, 2000). We concluded that exogenously administered NO could trigger the preconditioned state, but that endogenous production of NO was not involved in IPC. This conundrum was not resolved for several years until we repeated studies with L-NAME in IPC in *in vivo* rabbit hearts (Cohen *et al.*, 2006). L-NAME blocked the protection of IPC. Our earlier observations, although accurate, were dependent on the *in vitro* model used. As seen in Figure 1, bradykinin, opioids and adenosine are released by the ischaemic heart. But in the isolated, buffer-perfused heart, the absence of circulating kininogens would minimize release of bradykinin. In addition, opioid release would be attenuated because of the absence of cardiac innervation. Therefore, virtually all triggering would be the result of adenosine release which bypasses the NO-dependent trigger pathway (Figure 1).

As noted earlier, classical signalling dogma indicates that NO stimulates GC leading to generation of cGMP which in turn activates PKG (Figure 1). Studies with activators and inhibitors of PKG and cGMP analogues (Han *et al.*, 2002; Oldenburg *et al.*, 2004; Qin *et al.*, 2004; Kuno *et al.*, 2008) clearly demonstrated the involvement of PKG in IPC, and Baxter's laboratory (D'Souza *et al.*, 2003; Burley *et al.*, 2007) demonstrated increased myocardial levels of cGMP after protection by B-type natriuretic peptide. Additionally, BAY 58-2667, a NO-independent GC activator, conditioned rat and rabbit hearts (Krieg *et al.*, 2009). Thus, in addition to proven involvement of endogenous NO, there is much evidence to support participation of GC and PKG in conditioning's protection.

Several investigators have also demonstrated involvement of a NO-mediated, PKG-independent signalling pathway (Sun *et al.*, 2013; Penna *et al.*, 2014). NO can directly modify sulfhydryl residues by S-nitrosylation. The latter is an important post-translational protein modification in signalling. IPC increases S-nitrosylation and IPC cardioprotection can be aborted by treatment with ascorbate which is a reducing agent resulting in specific degradation of S-nitrosylated compounds. Additionally, in isolated mouse hearts, pharmacologic inhibition of the soluble GC/cGMP/PKG pathway failed to block IPC-induced cardioprotection. Thus, in at least some models, this alternative pathway of NO signalling may be important and it is possible that each pathway may contribute to cardioprotection and the ischaemic stimulus itself or other unidentified factors may determine whether one or the other pathway is utilized. Once again, there is redundancy to the response to ischaemia which may be an adaptive change insuring a maximal cardioprotective result.

ATP-sensitive potassium channels and redox signalling

The next critical step in this signalling cascade is opening of an ATP-sensitive K^+ channel (K_{ATP}). At the same time that we were uncovering the importance of adenosine in IPC, Garret Gross' laboratory was doing studies with K_{ATP} channels. Those investigators found that glibenclamide, a blocker of the channel, could also selectively block IPC's protection (Gross and Auchampach, 1992). Similarly, pretreatment with a K_{ATP} channel opener mimicked the protection (Gross and Auchampach, 1992). For a while it seemed that it had to be either adenosine or potassium channels which governed protection, but it turned out that they were simply two links in the same chain; both were involved. Several subsequent studies revealed that the K_{ATP} channel in question was located not on the sarcolemma but in the inner mitochondrial membrane (Garlid *et al.*, 1997; Liu *et al.*, 1998).

Mitochondrial K_{ATP} channels (mtK_{ATP}) are not triggers for IPC but rather are a critical link in the signalling pathway between surface receptors and PKC (Pain *et al.*, 2000). Opening of mtK_{ATP} is PKG-dependent (Costa *et al.*, 2005; 2008), but the channels are obviously not accessible to cytosolic PKG. There are intermediate steps involving PKCε in the mitochondria which transmits the signal from cytosolic PKG to the mtK_{ATP} channel (Costa *et al.*, 2005; 2008; Jabůrek *et al.*, 2006). One theory proposes that PKG reaches the mitochondria via signalosomes that bud off of sarcolemmal caveolae and contain critical signalling enzymes (Garlid *et al.*, 2009). Channel opening permits K^+ to enter the matrix along its electrochemical gradient. K^+ influx is balanced by electrogenic H^+ efflux driven by the respiratory chain.

An important link in this signalling is the redox coupling of mtK_{ATP} channel opening and PKC activation. Forbes *et al.* (2001) were the first to recognize this link when they noticed that either of the antioxidants N-acetylcysteine or N-2-mercaptopropionylglycine could block the protection from the mtK_{ATP} opener, diazoxide. It is not known exactly how mtK_{ATP} opening causes production of free radicals, but one theory is that mtK_{ATP}-dependent matrix alkalinization affects complex I and/or III which are poised to generate increased amounts of superoxide and its products H_2O_2 and hydroxyl radical (Costa and Garlid, 2008). All of the signalling steps to this point occur in ischaemic cells. However, generation of this burst of reactive oxygen species (ROS) must await reintroduction of oxygen into the myocardium which occurs during the reflow phase of the preconditioning cycle of ischaemia/reflow. There are many PKC isozymes. It appears that activation of PKCε is necessary and sufficient to achieve cardioprotection, while activation of PKCδ specifically blocks protection (Dorn *et al.*, 1999; Ping *et al.*, 2002; Inagaki *et al.*, 2003a,b). Thus, activation of PKC continues the signalling cascade.

The relationship among mtK_{ATP}, ROS and PKC is poorly understood. While ROS can directly activate PKC by causing release of Zn^{++} from the regulatory domain (Korichneva *et al.*, 2002), connexin 43 (Cx43) appears to be a vital link in redox signalling. Cx43 which makes most of the gap junctions between cardiomyocytes was noted to be necessary for preconditioning's protection (Schwanke *et al.*, 2002). It was later noted that protection depended on a mitochondrial population of Cx43 hemichannels located on the inner membrane. Depletion of these channels attenuates both protection and ROS production from an mtK_{ATP} opener (Heinzel *et al.*, 2005). Most recently, it was shown that an mtK_{ATP} opener causes phosphorylation of Cx43 by PKC and that phosphorylation is

required for protection (Srisakuldee *et al.*, 2009). This suggests some sort of circular signalling circuit as phosphoCx43 is needed for ROS production and ROS cause PKC activation, but PKC phosphorylates Cx43. The role actually played by Cx43 in the protective process (e.g. a channel, a signalling molecule or a scaffold) is still a mystery.

The redox signalling step explains one of the mysteries of IPC. Why is the heart protected when a prolonged ischaemic period is preceded by a short coronary occlusion followed by reperfusion but yet is not protected during a single prolonged insult? All of the trigger receptors are activated during the single prolonged ischaemic insult, but signalling stops at the step requiring redox coupling to PKC because of the lack of oxygen which is supplied during IPC's short reperfusion. A ROS scavenger blocks protection from IPC and it can easily be seen that the critical time for that blockade is during IPC's reperfusion phase (Dost *et al.*, 2008). While ROS-sensitive dyes indicate that radical production can occur during ischaemia (Becker *et al.*, 1999), apparently the ROS species generated is not one capable of the redox signalling. We also have found that reperfusing with hypoxic perfusate during the preconditioning protocol abrogates IPC's protection (Dost *et al.*, 2008). The identity of the ROS species involved has not been positively identified but seems to be a downstream product of HO· and is likely a product of phospholipid oxidation (Garlid *et al.*, 2013).

Adenosine signalling

Signalling initiated by the third endogenous agonist that triggers IPC, adenosine, is different. Adenosine's cardioprotective effect is not dependent on Src tyrosine kinase or PI3K (Qin *et al.*, 2003). Adenosine signalling seems to completely bypass mtK_{ATP} and ROS production (Cohen *et al.*, 2001) and it more directly activates PKC (see Figure 1) which is where all of the trigger signalling converges. The adenosine A_1 receptor is coupled through G_i to PLC and PLD. After the ligand binds to the receptor, G_i is cleaved into α and βγ moieties which activate PLC in the sarcolemma. This enzyme catalyzes the hydrolysis of membrane inositol-containing phospholipids, including phosphatidylinositol 4,5-bisphosphate. The resulting DAG stimulates translocation and activation of PKC. PLD also increases DAG levels by degrading phosphatidylcholine into choline and phosphatidic acid and the latter is transformed by a phosphohydrolase into DAG. These phospholipid activators of PKC also trigger release of zinc from PKC's regulatory domain (Korichneva *et al.*, 2002). The diversity of signalling among the triggers is confusing, but also reassuring. The redundancy ensures cardioprotection even if one or more elements in the triggering cascade are blocked.

The mediator phase

All signalling to this point occurs during the preconditioning cycles of ischaemia and reflow. These steps are collectively called the trigger phase. Subsequent steps, and there are several, are part of the mediator phase which occurs following termination of the prolonged period of ischaemia (the index ischaemia) with reperfusion (Figure 1).

A_{2B} receptors

It had been noted that adenosine receptors were required for IPC's protection in the mediator phase. One hypothesis suggested that preconditioning is protective by increasing tissue adenosine levels through activation of ecto-5′-nucleotidase (Kitakaze *et al.*, 1993). However, measurements of myocardial adenosine levels revealed that tissue adenosine concentration actually falls in IPC hearts (Goto *et al.*, 1996; Martin *et al.*, 1997). Our studies have indicated that the initial step of the mediator phase is activation of adenosine A_{2B} receptors (Philipp *et al.*, 2006). This receptor has a very low affinity for adenosine such that even during ischaemia when tissue adenosine levels reach 1–4 μM, this level would still be well below the A_{2B} adenosine receptor's K_D of 5–15 μM. However, PKC activation appears to raise the affinity of the A_{2B} receptor permitting the adenosine concentration in ischaemic myocardium to be sufficient for occupation of this receptor (Kuno *et al.*, 2007). It had already been shown that PKC activity can sensitize A_{2B} signalling, although no physiologic significance was attributed to the observation (Nordstedt *et al.*, 1989; Nash *et al.*, 1997; Trincavelli *et al.*, 2004). Although the details of this sensitization are still unknown, it would appear A_{2B} receptors can respond to the heart's endogenous adenosine only after this sensitization. Thus, we proposed that the affinity state of the A_{2B} receptor is the determinant that distinguishes the preconditioned from the non-preconditioned phenotype. Our observation of involvement of the A_{2B} receptor in IPC was supported by Eckle *et al.* (2007) who studied mice genetically modified to lack one of the four adenosine receptor subtypes. While A_1, A_{2A} and A_3 adenosine receptor knockout mice could be preconditioned, A_{2B} knockout mice could not. However, there is evidence suggesting a cooperative role of A_{2A} and A_{2B} adenosine receptors in some forms of cardioprotection (Xi *et al.*, 2009; Methner *et al.*, 2010).

The reperfusion injury survival kinases (RISK) pathway

A kinase cascade involving PI3K, Akt and ERK has been proposed to occur in the first minutes of reperfusion following the index ischaemia (Hausenloy and Yellon, 2004; Hausenloy *et al.*, 2005). These kinases have collectively been termed RISK (Hausenloy and Yellon, 2004). Although RISK are clearly involved in cardioprotection in rat (Hausenloy *et al.*, 2005) and rabbit (Yang *et al.*, 2004b; 2005) hearts, their involvement may not be universal. In pig hearts, RISK are less important (Skyschally *et al.*, 2009a). A distinct alternate pathway utilizing membrane TNF-α receptors and cytoplasmic JAK and STAT has been proposed (see succeeding text), although the end-effector for this and the RISK pathways appears to be identical.

IPC's end-effector

IPC's end-effector appears to be the mitochondrial permeability transition pore (mPTP) and its inhibition is considered to be the final step in the protective signal transduction pathway (Griffiths and Halestrap, 1993; 1995; Squadrito *et al.*, 1999; Di Lisa *et al.*, 2001; Hausenloy *et al.*, 2002). Although the molecular structure of mPTP is controversial, when

formed it is a high conductance pore in the inner mitochondrial membrane that dissipates the transmembrane proton/electrochemical gradient that drives ATP generation. The presence of the pore would logically lead to ATP depletion, enhanced ROS production, failure of membrane ion pumps, solute entry, organelle swelling and ultimate mitochondrial rupture. Destruction of large numbers of mitochondria will result in necrosis of the cardiomyocyte. Importantly, mPTP formation is inhibited by acidosis and promoted by calcium and ROS. The low pH during ischaemia inhibits transition pore formation. But restoration of pH coupled with rapid elevation in mitochondrial calcium and ROS cause the pores to form soon after reperfusion.

The cardioprotective signalling pathways keep mPTP closed. In the RISK pathway, there is involvement of an additional intervening kinase, glycogen synthase kinase-3β (GSK-3β) (Tong *et al.*, 2002; Gross *et al.*, 2004; Juhaszova *et al.*, 2004). This kinase is likely the final cytoplasmic kinase in IPC's signal transduction pathway. Interestingly, preconditioning leads to Ser^{9} phosphorylation and inhibition, not activation, of this kinase. Thus, GSK-3β inhibition blocks mPTP formation. Accordingly, GSK-3β inhibitors given at reperfusion mimic preconditioning (Förster *et al.*, 2006).

Alternative signalling pathways

As already noted, the importance of RISK has been clearly demonstrated in rat (Hausenloy *et al.*, 2005) and rabbit (Yang *et al.*, 2004b; 2005) hearts, but their involvement may not be required in all species. In a well-established pig heart model, activation of RISK was not increased by ischaemic postconditioning (IPoC), protection mediated by several brief reocclusions after release of the prolonged index coronary occlusion, over that seen in control hearts without postconditioning (Skyschally *et al.*, 2009a). Furthermore, wortmannin, a potent antagonist of PI3K, could not abort postconditioning's protection (Skyschally *et al.*, 2009a). In response to this conundrum, additional investigations uncovered another signalling pathway not dependent on RISK.

Survivor activating factor enhancement (SAFE) pathway

The SAFE pathway has been established, at least in rodent (Lecour *et al.*, 2005b; Lacerda *et al.*, 2009; Lecour, 2009) and porcine (Bhatt *et al.*, 2013) hearts. However, a caveat is important. Some individual signalling steps have been identified, but a roadmap or signal transduction pathway as developed for the RISK pathway (Figure 1) is not yet available. So evidence supporting involvement of the SAFE pathway is more fragmentary, even if compelling.

TNF-α signalling

The cytokine TNF-α is an important endogenous cardioprotectant released by IPC (Smith *et al.*, 2002; Lecour, 2009) and IPoC (Lacerda *et al.*, 2009), possibly as part of the myocardial inflammatory response during reperfusion. TNF-α knockout mice cannot be protected by either IPC (Smith *et al.*, 2002; Lecour, 2009) or IPoC (Lacerda *et al.*, 2009; Lecour, 2009), whereas low-dose exogenous TNF-α in lieu of ischaemia can both precondition (Smith *et al.*, 2002; Lecour *et al.*, 2005b; Lecour, 2009) and postcondition (Lacerda *et al.*, 2009; Lecour, 2009) hearts. As seen with the G_i-coupled receptor triggering described earlier for IPC, TNF-α preconditioning of rat hearts can be abolished by the antioxidant N-2-mercaptopropionyl glycine (Lecour *et al.*, 2005a), a ROS scavenger, 5-hydroxydecanoate (Lecour *et al.*, 2002), an antagonist of mtK_{ATP}, and chelerythrine (Lecour, 2009), a PKC antagonist, implying free radicals, mtK_{ATP} channels, and PKC play important roles. However, further information about the downstream effect of ROS, opening of mtK_{ATP}, or PKC or their targets is not available. Interestingly, TNF-α's effect on ischaemic myocardium is concentration-dependent. High doses of TNF-α are not protective and may actually increase infarct size (Lecour *et al.*, 2002; Lecour, 2009).

There are two TNF receptor isoforms, TNFR1 and TNFR2. Exogenous TNF-α confers cardioprotection in TNFR1 (also known as TNFRSF1A) but not TNFR2 (also known as TNFRSF1B) knockout mice, thereby implying it is TNFR2 which is responsible for the ligand's cardioprotective effect (Lacerda *et al.*, 2009). TNF-α administered as either a pre- or postconditioning-mimetic does not lead to phosphorylation of either Akt or ERK, and neither PD98059 nor wortmannin, antagonists of the ERK and PI3K pathways, respectively, can abort the protection of exogenous TNF-α. The SAFE pathway's downstream signalling is, therefore, not dependent on these traditional RISK (Lecour *et al.*, 2005b; Lacerda *et al.*, 2009). In contrast, TNF-α, IPC and IPoC all phosphorylate STAT3 and the protective effect of pharmacological pre- and postconditioning with TNF-α is abolished by AG490, an inhibitor of STAT3 (Lecour *et al.*, 2005b; Lacerda *et al.*, 2009). JAKs are a family of tyrosine kinases associated with the cytoplasmic domains of cytokine and growth factor receptors, for example, IL-6, growth hormone and TNFR2. After the TNF-α ligand binds to its receptor, two adjacent JAKs are transphosphorylated and subsequently activate STAT proteins by phosphorylation. Tyrosine-phosphorylated STAT proteins form homo- and heterodimers that translocate to the nucleus where they influence gene transcription, especially of stress-responsive genes (Levy and Lee, 2002; Myers, 2009). Serine-phosphorylated STAT translocates to mitochondria to regulate electron transport (Myers, 2009; Wegrzyn *et al.*, 2009). Although STAT3 is by definition a transcription factor, its effects in ischaemia/reperfusion are much too rapid to assume that it is working by modulating gene transcription. Therefore, it must have additional direct effects. It appears to protect by phosphorylating and, therefore, inactivating GSK-3β (Lacerda *et al.*, 2009; Pedretti and Raddatz, 2011), also a downstream target in the RISK pathway. Thus, the RISK and SAFE pathways appear to converge on the same targets. In fact there is some evidence of cross-talk between these two pathways, so they may not be totally independent (Lecour, 2009; Somers *et al.*, 2012). Additionally, IPoC in pigs increased tyrosine phosphorylation of mitochondrial STAT3 which improves complex I respiration and calcium retention capacity (Heusch *et al.*, 2011). Inhibition of JAK/STAT blocks both increased phosphorylation of mitochondrial STAT3 and the cardioprotective effect of IPoC. Mitochondrial STAT3 co-immunoprecipitates with cyclophilin D, the target of the mPTP inhibitor cyclosporin A and, therefore, could inhibit pore formation (Boengler *et al.*, 2010).

Sphingosine is a trigger for RISK and SAFE

Sphingosine is a membrane sphingolipid which is catalyzed to sphingosine 1-phosphate (S1P) by two sphingosine kinase (SPHK) isoforms, SPHK1 and SPHK2. It is the former that is associated with cell survival. S1P is released in both IPC and IPoC (Karliner, 2013). Many S1P actions are mediated through S1P GPCR subtypes. The $S1P_1$ receptor is most prominently expressed in cardiomyocytes. The $S1P_1$ receptor couples to $G\alpha_i$. $S1P_2$ and $S1P_3$ receptors are also present on cardiomyocytes and couple to both $G\alpha_q$ and $G\alpha_i$. Binding of the ligand S1P to the $S1P_1$ receptor leads to downstream activation of ERK1/2 and $S1P_3$ receptor binding results in activation of PI3K and Akt. Thus, S1P cardioprotection in part depends on RISK (Knapp, 2011; Somers *et al.*, 2012). However, S1P cardioprotection is also dependent on the SAFE pathway through the $S1P_2$ receptor which activates ERK1/2 and subsequently STAT3 (Knapp, 2011; Somers *et al.*, 2012). As already noted, multiple pathways provide potential for robust protection.

Sphingosine intermediates are also involved in cardioprotection mediated by TNF-α. The latter's protective effect is attenuated in the presence of inhibitors of the sphingolipid pathway (Lecour *et al.*, 2002). TNF receptor-associated factor 2 (TRAF2) is a downstream target of TNFR2. TRAF2 can activate intracellular formation of S1P by up-regulating SPHK1 (Frias *et al.*, 2012). Also, S1P activates STAT3.

This redundancy of pathways probably enhances the potential survival of the cell. Blockade of any one pathway does not lead to inevitable death of the cell. It is still possible for an alternate pathway to provide some protection. Alternatively, we may simply be looking at isolated sections of a larger complex integrated system that we still do not fully appreciate. This may be analogous to the fable of the blind men describing an elephant based only on the part of the animal they were touching.

Genesis of reperfusion therapy

Hence, IPC is a potent cardioprotective intervention that is the result of a complex, two-phase signalling pathway leading to inhibition of mPTP formation. However, the obvious drawback is that IPC by definition must be instituted prior to the onset of ischaemia. In patients presenting to the hospital with an AMI, ischaemia is already ongoing and preconditioning is not possible. However, Hausenloy *et al.* (2005) proposed that if IPC, an intervention introduced before the onset of ischaemia, protects by inducing activation of the RISK pathway at reperfusion, then it should still be possible to activate this pathway during ischaemia and still effect salvage of myocardium. This revolutionary paradigm shift provided hope that IPC could be translated into a meaningful clinical intervention by focusing on early reperfusion. Indeed, multiple reagents were found to protect the myocardium when given in the first minutes of reperfusion, for example, insulin (Baines *et al.*, 1999), the adenosine A_1/A_2 agonist AMP579 (Xu *et al.*, 2000), the A_{2B} adenosine receptor-selective agonist BAY 60-6583 (Albrecht *et al.*, 2006), TGFβ1 (Baxter *et al.*, 2001), urocortin (Schulman *et al.*, 2002), cardiotrophin-1 (Liao *et al.*, 2002), adenosine agonist 5′-(N-ethylcarboxamido) adenosine (Yang *et al.*, 2004a), bradykinin (Yang *et al.*, 2004a), natriuretic peptides (Baxter, 2004; Yang *et al.*, 2006a), erythropoietin (Cai and Semenza, 2004; Parsa *et al.*, 2004) and cyclosporin A (Hausenloy *et al.*, 2009). All depend on activation of PI3K and/or ERK except for cyclosporin A which is a direct inhibitor of mPTP formation.

Clinical trials

Not surprisingly, several clinical trials of proposed IPC-mimetics have been completed, although none has been greatly successful. These trials require study of many patients and are expensive. Repeated failures have left the pharmaceutical companies quite leery. Two large clinical trials, Amistad I (Mahaffey *et al.*, 1999) and II (Ross *et al.*, 2005), were organized to evaluate the effectiveness of adenosine. In the first trial, all patients with AMI were evaluated, whereas in the second trial, patients with only higher risk anterior infarcts were included. In Amistad I, there was no difference between the control and adenosine-treated subjects, although a *post hoc* analysis suggested that data in the subgroup with anterior infarcts looked promising (Birnbaum *et al.*, 2002). In Amistad II, results were again disappointing. Smaller infarcts were noted in only a high-dose subgroup, but clinical outcomes were not improved. Although adenosine plays an important role in preconditioning as both a trigger and a mediator, the Amistad trials used low-dose i.v. infusion of adenosine which in preclinical studies had clearly not been universally successful at protecting ischaemic myocardium (Olafsson *et al.*, 1987; Goto *et al.*, 1991; Norton *et al.*, 1991; 1992; Pitarys *et al.*, 1991; Velasco *et al.*, 1991; Vander Heide and Reimer, 1996; Budde *et al.*, 2000). It was probably not advisable to undertake such large and expensive trials until the cause of the discrepant data had been identified and resolved. Adenosine's hypotensive side effect limits the concentration that can be administered parentally and we were unable to precondition rabbit hearts with the highest dose of i.v. adenosine they would tolerate (Liu *et al.*, 1991). We could, however, condition them with receptor-selective adenosine analogs such as AMP579 (Xu *et al.*, 2003).

Atrial natriuretic peptide activates PKG in cardiomyocytes and mimics IPC when injected just prior to reperfusion in animals (Yang *et al.*, 2006a). It produced a statistically significant, but very modest, reduction in infarct size and a similarly modest increase in ejection fraction (Kitakaze *et al.*, 2007). The disappointingly modest effect might be explained by failure to stratify patients into low- and high-risk groups. The size of a patient's ischaemic zone is dependent on the location in the coronary artery where the thrombus forms. Studies from Ovize's laboratory (Staat *et al.*, 2005) indicate that in patients with AMI who are reperfused with primary angioplasty, those with small ischaemic zones have very small infarcts and virtually complete recovery regardless of treatment. Including these patients in the analysis greatly dilutes the potential significance of any intervention. Therapeutic benefit can best be appreciated in the subgroup of high-risk patients presenting with large ischaemic zones. Other possible reasons for the modest result are discussed in the succeeding text.

During ischaemia, pH falls as H^+ accumulates. As a result, the Na^+/H^+ exchanger (NHE) is activated and Na^+ exchanges for H^+ in a 1:1 stoichiometric manner. In turn, the Na^+/Ca^{2+}

exchanger will transport Na^+ out of the cell in favour of Ca^{2+}. These ionic movements are magnified during reperfusion when restored blood flow quickly normalizes the pH of the extracellular fluid. The resulting high intracellular Ca^{2+} concentration should encourage mPTP formation. It was decided to evaluate NHE blockers cariporide (Théroux *et al.*, 2000; Mentzer *et al.*, 2008) and eniporide (Zeymer *et al.*, 2001) in large clinical trials despite preclinical investigations which demonstrated efficacy only when the drug was administered before ischaemia (Miura *et al.*, 1997). In the first trial of cariporide, GUARDIAN, patients studied had unstable angina pectoris, non-ST-segment elevation myocardial infarction, angioplasty or coronary revascularization surgery (Théroux *et al.*, 2000). Only those that had pretreatment, the surgical group, showed any benefit. A second trial, EXPEDITION, concentrated on coronary bypass patients, but the new study design inexplicably included prolonged infusions of cariporide which were associated with more strokes (Mentzer *et al.*, 2008). The increased stroke risk doomed further consideration of cariporide. Although eniporide, another NHE blocker, was evaluated in patients with acute ST-segment-elevation myocardial infarction (STEMI), no difference was observed (Zeymer *et al.*, 2001).

Investigations of other interventions which may have shown some promise in preclinical evaluations have also not been very successful. Thus, pexelizumab (APEX AMI Investigators *et al.*, 2007), an antibody to a complement component, erythropoietin (Cleland *et al.*, 2010), a stimulator of haematopoiesis in response to hypoxia, and delcasertib (Lincoff *et al.*, 2014), a selective inhibitor of PKCδ, all failed to meet the primary objectives of the trials, reduction of infarct size and improvement of the clinical status of the subjects. There is a lesson: clinical trials should probably not be undertaken until multiple preclinical laboratories have confirmed salutary effects of the intervention and until practical information about dosing and timing of administration has been established (Downey and Cohen, 2009).

A small proof-of-concept study of cyclosporin A in patients with AMI produced very encouraging results (Piot *et al.*, 2008). Patients were stratified by the size of their ischaemic zone and each received a bolus of cyclosporin A before recanalization. Those with the highest risk benefited the most from exposure to the drug. There are plans to repeat this investigation in a much larger cohort in Europe. However, as explained in the succeeding text, a second study may be problematic because concomitant use of antiplatelet drugs dictated by current standard of care considerations may mask cyclosporin's protection.

There have been other proof-of-concept studies examining commonly used agents. Van de Werf *et al.* (1993) studied the effect of atenolol administered prior to thrombolysis in patients with AMI. This β-blocker had no impact on infarct size, a result echoing that of a study in dogs by Reimer and Jennings (1984). Nonetheless, a very recent examination of i.v. metoprolol shortly before percutaneous coronary intervention (PCI) in patients with STEMI was encouraging (Ibanez *et al.*, 2013). This β-blocker modestly decreased infarcted myocardium as a percentage of risk zone from approximately 78% in untreated hearts to 68%. Although there was protection, small group sizes limit the significance of the conclusion and require conduct of a large, expensive clinical trial for confirmation. There is no evidence that β-adrenoceptor blockade triggers IPC signalling as detailed earlier. The same is true for exenatide, a glucagon-like peptide-1 (Lønborg *et al.*, 2012). IPC's signalling is not the only way to protect the heart against infarction, and hypothermia and early reperfusion are obvious examples.

IPoC

It was clearly understood that preconditioning cycles must be completed before initiation of ischaemia. Yet Hausenloy *et al.* (Hausenloy and Yellon, 2004; Hausenloy *et al.*, 2005) found that IPC actually exerted its protection at reperfusion. This finding led Vinten-Johansen *et al.* to test whether serial coronary occlusions after the index coronary occlusion/reperfusion might also protect the ischaemic heart. After many tries, they found that several (three) short (30 s) cycles of reperfusion/occlusion immediately after the initial reperfusion were almost as protective as IPC in an open-chest dog model (Zhao *et al.*, 2003). They called this IPoC. This seemingly improbable observation has been reproduced in many laboratories (Skyschally *et al.*, 2009b) and the ensuing protection was shown to be dependent on the same signals as IPC (Yang *et al.*, 2004b; 2005).

Again, the final effector for IPoC appeared to be prevention of mPTP formation (Argaud *et al.*, 2005; Gateau-Roesch *et al.*, 2006). Because of the widespread success in the experimental laboratory, a leap was made to the clinical arena. Patients with acute STEMI have thrombotic occlusion of a coronary artery. Standard treatment is recanalization, usually by mechanical aspiration or pulverization of the thrombus by percutaneous transluminal coronary angioplasty (PTCA). Opening of the occluded coronary artery by PTCA is equivalent to removing the ligature around the snared coronary artery in the experimental animal. For patients treated with primary angioplasty, IPoC could be accomplished by repeated balloon inflations to interrupt reflow for the postconditioning cycles. The initial report of IPoC in the cardiac catheterization laboratory was very encouraging (Staat *et al.*, 2005). Using a risk stratification design, they showed a highly significant reduction of infarct size in IPoC patients with large ischaemic zones. But why does staccato reperfusion lead to myocardial salvage?

In the non-conditioned naïve heart following coronary occlusion, mtK_{ATP} open during ischaemia, but there is no oxygen so the pathway is blocked at the redox signalling step. During ischaemia, mPTPs are inhibited by acidosis in the tissue probably by blocking calcium binding to cyclophilin and displacing the latter which is required for mPTP formation. Upon reperfusion, acids quickly wash away restoring pH to 7.4 and mPTP forms before PKC can be activated to trigger the remainder of the signalling pathway thus resulting in necrosis of the tissue. On the other hand, IPoC maintains some acidosis in the reperfused tissue because of the repeated occlusion periods while still allowing oxygenation during the reperfusion periods (Cohen *et al.*, 2007b; 2008). Reintroduction of oxygen while the tissue pH is still acidic allows the tissue to activate PKC through redox signalling while mPTP formation is still inhibited (Cohen *et al.*, 2007b; 2008). Once the PKC pathway is activated, the cell is able to inhibit mPTP

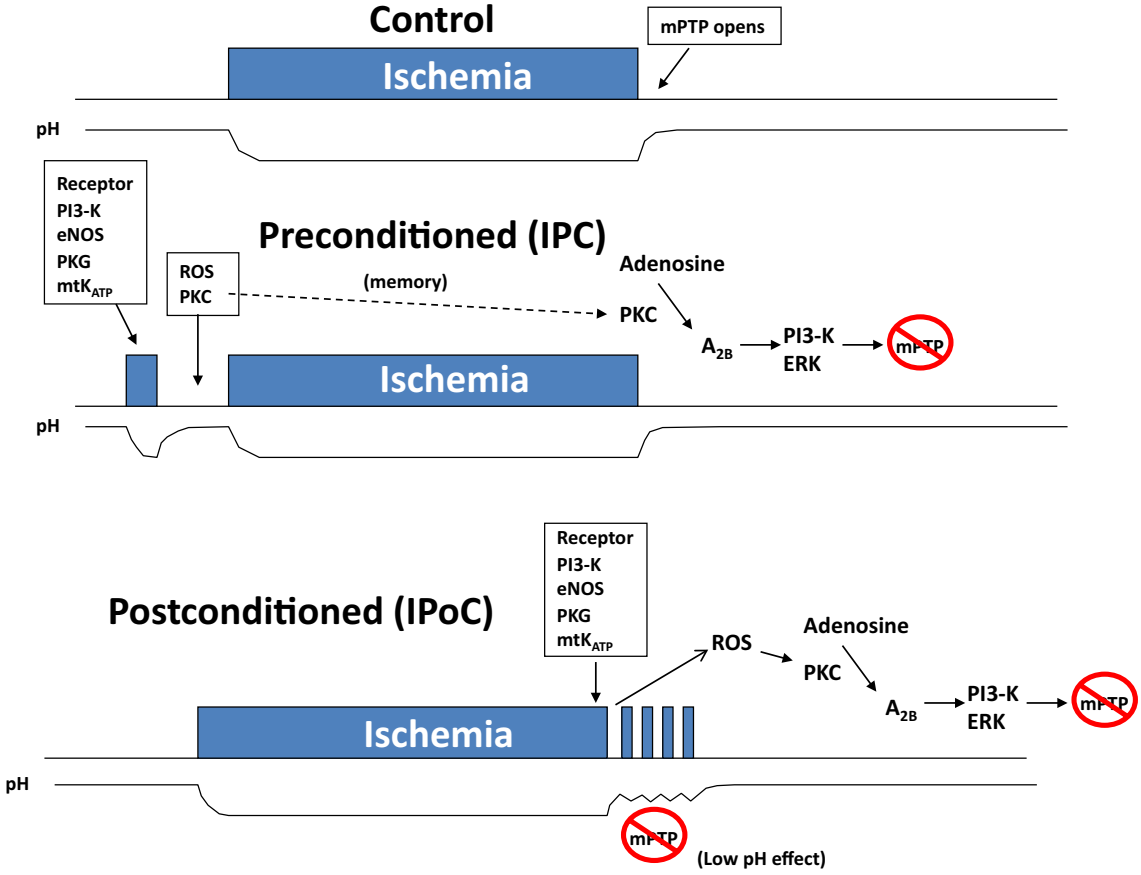

Figure 2

Signalling during ischaemic pre- and postconditioning and effect of pH and transient reoxygenation on that signalling and mPTP formation. See Figure 1 for abbreviations. Modified from Cohen *et al.* (2007b; 2008).

formation through the conditioning pathway that IPC uses even after pH is normalized, and hence necrosis is reduced.

Thus, there is a race between ROS-mediated activation of PKC leading to subsequent triggering of the remainder of the signal cascade and washout of mPTP-inhibiting H^+. Figure 2 summarizes the pH hypothesis of IPoC's protection. mPTPs in the naïve, non-conditioned heart (Figure 2, upper panel) are inhibited by the low pH during the ischaemic period. But as soon as reperfusion is permitted, H^+ is washed out and mPTPs open leading to tissue necrosis. In IPC (Figure 2, middle panel), signalling up to the opening of mtK_{ATP} occurs during the first brief ischaemic period. During the brief reperfusion oxygen is resupplied which leads to ROS generation and activation of PKC which then can sensitize adenosine receptors. Thus, at the beginning of reperfusion following the prolonged index coronary occlusion, RISK are activated and mPTPs are inhibited. The bottom panel of Figure 2 depicts events in IPoC. The initial signalling up to opening of mtK_{ATP} occurs during the prolonged period of ischaemia, but signalling cannot proceed until reperfusion when oxygen is reintroduced into the ischaemic tissue leading to generation of ROS. Because of the limited reflow, pH only partially recovers. The low pH inhibits mPTP formation long enough until redox signalling can lead to adenosine receptor population with sensitization and subsequent RISK signalling that results in inhibition of mPTP formation even after the muscle is fully reperfused.

The pH hypothesis is consistent with other observations made in experimental animals. Reperfusion interventions must be applied in the first minutes of reperfusion. Thus, delayed IPoC (Yang *et al.*, 2004b; Philipp *et al.*, 2005) or late infusion of the cardioprotective AMP579 (Xu *et al.*, 2003) leads to loss of the cardioprotective effects. Presumably, such delay would permit pH normalization before initiation of the intervention, thus allowing mPTP formation. Once this occurs, no intervention dependent for its success on keeping mPTP closed would be expected to salvage myocardium in the risk zone. Also, simply reperfusing the heart for several minutes with low pH buffer mimics IPoC (Cohen *et al.*, 2007b).

Conditioning with volatile anaesthetic agents

Shortly after IPC was recognized as a universally acknowledged cardioprotective strategy, volatile anaesthetic gases,

principally sevoflurane, isoflurane and desflurane were noted to also have cardioprotective abilities when applied in lieu of the brief cycles of ischaemia/reperfusion either before the prolonged index coronary occlusion (preconditioning) (Cope *et al.*, 1997) or following it (postconditioning) (Chiari *et al.*, 2005). The signalling steps are not as clearly defined as in IPC and IPoC, but it is fair to say there are many parallels. Basically, the volatile gases signal through adenosine and opioid receptors, modulate G proteins, stimulate PKC and other intracellular kinases, open mtK_{ATP} channels leading to ROS generation and activate RISK to keep mPTP from forming (Tanaka *et al.*, 2004; Chiari *et al.*, 2005; Feng *et al.*, 2005; Pravdic *et al.*, 2009). The gases may also have more direct effects on mtK_{ATP}. The potential clinical impact is obvious, although utility is limited to the surgical suite, either cardiac or non-cardiac (Swyers *et al.*, 2014).

Platelets and cardioprotection

As noted earlier, one of the earliest cardioprotective interventions applied clinically was IPoC. The initial clinical report was very encouraging (Staat *et al.*, 2005) and the intervention was adopted by many cardiac catheterization laboratories, partly because of the ease of application and partly because of the anticipated small likelihood of complications. Despite this early enthusiasm, other clinicians who attempted to replicate the positive results could not (Sörensson *et al.*, 2010; Freixa *et al.*, 2012; Tarantini *et al.*, 2012; Hahn *et al.*, 2013; Limalanathan *et al.*, 2014). Would this intervention have to join all of the other interventions which initially showed great promise but which did not live up to expectations and which failed to produce a significant and consistent beneficial clinical effect? Could there be a logical explanation for the inability of these later studies of IPoC to reproduce the early encouraging data?

Platelets are important cellular elements for initiation and propagation of a thrombus. It is now universally accepted that STEMI is caused by thrombosis and coronary occlusion following rupture of a plaque. Exposure of circulating platelets to collagen leads to their activation and accumulation. Platelet-activating factor (PAF) is a phospholipid which is released by neutrophils and monocytes during oxidative stress or ischaemia/reperfusion (Penna *et al.*, 2011). PAF is a chemoattractant for platelets and neutrophils and predisposes to capillary plugging and release of proteolytic enzymes and inflammatory mediators. Additionally, it causes coronary vasoconstriction. Cardiomyocytes also produce PAF and the latter's synthesis is triggered by ROS generation and oxidative stress at the beginning of reperfusion. PAF binds to receptors located on various cell types including smooth muscle cells, cardiomyocytes and endothelial cells. PAF receptor antagonists limit infarction in models of ischaemia/reperfusion (Montrucchio *et al.*, 1990; Ma *et al.*, 1992). Curiously, low doses of PAF are paradoxically cardioprotective (Penna *et al.*, 2005; 2011). The PAF receptor is a G_i protein-coupled receptor that triggers signalling similar to that seen in IPC (Figure 1).

Because the major risk of intracoronary dilatation and stenting is stent thrombosis and occlusion, antiplatelet drugs were tested as anticoagulants in patients undergoing primary angioplasty for AMI. Many clinical studies have demonstrated that antiplatelet agents do indeed improve prognosis of patients after AMI and minimize complications of stenting (Antiplatelet Trialists' Collaboration, 1994; Yusuf *et al.*, 2001; Sabatine *et al.*, 2005a,b; Wiviott *et al.*, 2007; Wallentin *et al.*, 2009; Bhatt *et al.*, 2013). This protection is particularly evident when the antiplatelet drug is given as a loading dose prior to the recanalization procedure. Thus, the COX antagonist aspirin, the thienopyridines clopidogrel and prasugrel and the triazolopyrimidines ticagrelor and cangrelor are very effective agents and all except cangrelor have won regulatory approval and have become standard of care in the treatment of patients with AMI or stenting. Aspirin blocks production of thromboxane by the platelet, while the thienopyridines and triazolopyrimidines block the platelet $P2Y_{12}$ ADP receptor and all effectively attenuate platelet aggregation. It has been assumed that it is the anti-aggregatory effect of these agents on platelets to minimize intravascular thrombosis that is responsible for their well-documented clinical benefits. However, that mechanism of action has not been unequivocally determined. In this regard, it is instructive to review some of the preclinical data.

Preclinical studies of antiplatelet drugs

Barrabés *et al.* (2010) noted that activated platelets from patients with AMI infused into isolated rat hearts before onset of ischaemia/reperfusion increased infarct size, whereas platelets from healthy volunteers had no effect. Furthermore, perfusion of previously ischaemic hearts with platelets activated in a second animal following ischaemia/reperfusion led to deterioration of left ventricular function and larger myocardial infarcts (Knight *et al.*, 2001; Mirabet *et al.*, 2002). Aggregation of platelets from mice with deficiencies of either platelet receptor glycoprotein (GP) VI (Takaya *et al.*, 2005; Li *et al.*, 2007) or signalling protein G_q (Weig *et al.*, 2008) is diminished, and infarcts are smaller following ischaemia/reperfusion. These observations suggest activated platelets have deleterious effects on ischaemic myocardium and provide some support for the hypothesis that there is a relationship between platelet aggregation and consequences of myocardial ischaemia/reperfusion, for example, infarct size.

Preclinical investigations of blockade of platelet aggregation in ischaemia/reperfusion have mostly studied effects of GPIIb/IIIa antagonists. An experimental GPIIb/IIIa inhibitor which abolished *in vitro* platelet aggregation decreased infarction in dogs undergoing ischaemia/reperfusion when it was administered before reperfusion (Kingma *et al.*, 2000). However, Kingma *et al.* (2000) also noted that the platelet antagonist had no effect on myocardial blood flow during reperfusion, and therefore, postulated that this infarct-sparing action was not related to blood flow but rather was the result of a direct protective effect on heart muscle. This was the first suggestion of a direct cardioprotective effect by an inhibitor of platelet aggregation.

Kunichika *et al.* (2004) made similar observations in dogs treated with tirofiban, a GPIIb/IIIa antagonist. However, this agent which decreased infarct size also increased myocardial blood flow within the risk area. Consequently, the investigators attributed the agent's cardioprotection to improvement in microvascular flow. Tirofiban also decreased the area of no-reflow in pigs during reperfusion and decreased infarct size (Yang *et al.*, 2006b). In dogs with coronary thrombosis

treated with angioplasty, tirofiban improved myocardial blood flow following reperfusion, decreased the size of the no-reflow zone and made infarcts smaller (Sakuma *et al.*, 2005). It was assumed that inhibition of platelet aggregation protected by preventing microthromboembolism.

Additional studies failed to corroborate this hypothesis. The deleterious effect of the addition of activated pig platelets to perfused, isolated rat hearts subjected to ischaemia/reperfusion was not blocked by tirofiban (Mirabet *et al.*, 2002). A second GPIIb/IIIa inhibitor had no effect on infarct size in a porcine model of ischaemia/reperfusion (Barrabés *et al.*, 2002). In both studies, platelet aggregation was blocked by the GPIIb/IIIa antagonists. It is not known why these latter studies differed from the former.

$P2Y_{12}$ receptor inhibitors may be postconditioning agents

Because of this confusion, we evaluated a variety of platelet inhibitors in rabbits undergoing 30 min coronary occlusion/3 h reperfusion (Yang *et al.*, 2013c). For most of the studies, we examined the effects of cangrelor, an i.v. agent which could be administered minutes before reperfusion and which would have immediate effects. Oral agents suffer from the limitations imposed by intestinal absorption and the requirement for conversion of the administered pro-drug clopidogrel or prasugrel to active metabolites. This delay causes an uncertainty of timing of onset of biological effect. A cangrelor bolus of 60 $\mu g \cdot kg^{-1}$ followed by an infusion of 6 $\mu g \cdot kg^{-1} \cdot min^{-1}$ attenuated platelet aggregation by more than 94%. Cangrelor resulted in an impressive decrease in infarct size from 38% of the risk zone in control rabbits to 19%, similar to the degree of protection seen after IPoC. Delay in cangrelor administration until 10 min after release of the coronary occlusion led to abrogation of protection, similar to that seen with delayed IPoC (Yang *et al.*, 2004b; Philipp *et al.*, 2005).

As described earlier, IPoC's protection is known to depend on a complex signal transduction pathway. Accordingly, we tested seven inhibitors of IPoC's signalling and protection: wortmannin and LY294002 (PI3K/Akt antagonists), PD98059 (antagonist of MAPK kinase 1/2 and therefore, ERK 1/2), 5-hydroxydecanoic acid (putative blocker of mtK_{ATP}), 8-(p-sulfophenyl) theophylline (non-selective antagonist of adenosine receptors), MRS 1754 (selective antagonist of adenosine A_{2B} receptors) and N-2-mercaptopropionylglycine (scavenger of ROS and blocker of redox signalling). All abolished cangrelor's protection. However, importantly, none restored platelet reactivity. Therefore, cangrelor's anti-aggregatory effect was still intact, but its cardioprotective action was totally blocked.

Hence, IPoC and cangrelor have identical kinase and receptor 'fingerprints' and this conclusion strongly supports the contention that the signalling and mechanism of protection of the two interventions are the same. We also determined whether the combination of cangrelor and IPoC would have an additive protective effect. It did not, further supporting the assumption that both induce protection by the same mechanism (Yang *et al.*, 2013c). Of course, to make this conclusion, it is critical that the effect of each individual intervention is maximized. Obviously, there would be an additive effect of two agents using the same pathway if one or both was used at a submaximal concentration. Hence, we propose that cangrelor is a bona fide conditioning agent and signal transduction rather than any effect on thrombosis causes the protection. Yet, we found that the magnitude of protection was correlated with the degree of suppression of aggregation indicating that $P2Y_{12}$ blockade was common to both processes.

Cangrelor in both primate (Yang *et al.*, 2013b) and rodent (Yang *et al.*, 2013a) hearts was also very protective when administered just before reperfusion. Cangrelor's protection was equivalent to that of IPoC in macaque hearts (Yang *et al.*, 2013b) and IPC in rat hearts (Yang *et al.*, 2013a). In monkeys, an antibody to platelet GPVI also decreased infarct size, indicating another intervention which decreased platelet aggregation and infarction (Yang *et al.*, 2013b).

Clopidogrel, a widely used $P2Y_{12}$ antagonist in patients with AMI and PTCA, was fed to rabbits for 2 days and it blocked platelet aggregation by 78% (Yang *et al.*, 2013c). Clopidogrel-treated animals also had significantly smaller infarcts than untreated rabbits. Wortmannin and MRS 1754 each abolished the protective effect, but the drug's anti-aggregatory action remained intact.

Finally, ticagrelor, a third platelet $P2Y_{12}$ receptor antagonist, administered by gavage to rats 2 h before coronary occlusion, decreased infarction from 45% of the risk zone in control animals to 26% (Yang *et al.*, 2013a). This protective effect was predictably blocked by wortmannin and PKC antagonist chelerythrine. Neither blocker interfered with ticagrelor's inhibitory effect on platelet reactivity.

Therefore, several pharmacologic and biologic interventions that blocked platelet aggregation also spared ischaemic myocardium from infarcting. It is critical to realize that although an antiplatelet effect linked all of the agents, inhibition of platelet activity was not the determining factor for cardioprotection. These appear to be true conditioning agents. Cangrelor in isolated rabbit hearts perfused with platelet-free Krebs buffer was not protective, suggesting that some blood element, presumably platelets, is somehow involved. Indeed, cangrelor's protective effect was lost in rats made thrombocytopenic with anti-thrombocyte serum (unpublished observation).

Are today's patients already postconditioned by loading doses of antiplatelet drugs?

In addition to the possible confounding effects of co-morbidities in man, generally absent in animal models, these experimental data on antiplatelet interventions, specifically $P2Y_{12}$ receptor antagonists, may help to explain the observations of protection or lack of protection following clinical IPoC. Because $P2Y_{12}$ antagonists are conditioning agents, they themselves would already be protecting the heart. Therefore, addition of a second intervention as IPoC which protected by the same mechanism would be expected to have little additional effect, similar to our observations in rabbits in which cangrelor and IPoC together were no more protective than either alone (Yang *et al.*, 2013c). In the later clinical studies of IPoC, virtually, all patients had received clopidogrel before the intervention (Sörensson *et al.*, 2010; Freixa *et al.*, 2012; Tarantini *et al.*, 2012; Hahn *et al.*, 2013; Limalanathan *et al.*, 2014), thus marking the patients as protected and perhaps dooming IPoC to be a superfluous and unneeded intervention. However, in the initial study by

Ovize's group in which patients with AMI were recruited prior to 2005, only about half had been premedicated with clopidogrel (Staat *et al.*, 2005). At our urging, these investigators went back to their database and segregated patients according to pre-IPoC administration of clopidogrel (Roubille *et al.*, 2012). Control patients treated with clopidogrel had smaller infarcts than those who were untreated, supporting our conclusion that clopidogrel is a cardioprotective agent. But these authors also noted that the combination of clopidogrel and IPoC salvaged more tissue than IPoC, contrary to our observations in rabbits. However, it is likely that neither intervention, clopidogrel nor IPoC, was optimized to fully condition the hearts when applied individually in contrast to our ability to maximize the effect of each intervention in animal models. Most of the patients in Roubille's retrospective analysis (Roubille *et al.*, 2012) had received only 300 mg of clopidogrel which is clearly suboptimal (Patti *et al.*, 2011). Also, the optimal postconditioning protocol for human hearts is unknown and only one protocol was tested. If neither intervention was optimal, an additive effect would be expected.

Piot *et al.* (2008) reported a significant reduction in infarct size by cyclosporin A in patients undergoing PCI. However, those patients were recruited by the same investigators as noted earlier (Staat *et al.*, 2005) and around the same time. The authors have not disclosed the clopidogrel usage in that cohort.

These observations on the effect of clopidogrel and other $P2Y_{12}$ receptor inhibitors are more than academic. Use of these agents in patients with AMI is now standard of care and their dosing has been optimized so that most of today's patients may already be postconditioned at the time of PTCA. Hence, any additional intervention using the same protective mechanism as the $P2Y_{12}$ receptor inhibitor would add little to the protection. This problem is compounded by many recent clinical trials that have led to incremental improvements in antiplatelet treatment. New blockers have greatly shortened absorption times and their dosing is being optimized so that eventually all patients presenting with AMI are likely to be optimally postconditioned. To obtain additional cardioprotection, an adjunct intervention must have a different mechanism of action.

Additional cardioprotection in the presence of an antiplatelet drug

We have found that some protective interventions can be combined to produce more potent protection. Unlike IPoC and cangrelor, mild hypothermia (Miki *et al.*, 1998b; Tissier *et al.*, 2007b) and cariporide (Miura *et al.*, 1997) are most protective when applied during ischaemia rather than reperfusion. Thus, protection from IPC which protects against a reperfusion injury could be added to that of cooling which protects against an ischaemic injury (Miki *et al.*, 1998b). Similarly, the protective effect of the pharmacological postconditioning agent AMP579 which protects against a reperfusion injury (Xu *et al.*, 2003) could be added to that of cariporide that protects against an ischaemic injury (Xu *et al.*, 2002).

We tested whether any interventions could be additive with cangrelor treatment (Yang *et al.*, 2013a). In rats, peritoneal lavage with ice-cold saline 10 min before coronary occlusion lowered blood temperature to 32–33°C and decreased infarction to 25% of the risk zone, equivalent to that seen with cangrelor treatment just before reperfusion. The two interventions together halved infarction to 14% of myocardium at risk. Cariporide's protective effect (27% infarction) is comparable with cangrelor's. The combination of cangrelor and cariporide nearly halved infarction to 16% of the risk zone. When all three interventions were combined, infarction again halved to only 6% of the risk zone. Obviously, treatment during the ischaemic period is logistically problematic. However, most patients spend an extended period of time with healthcare professionals before recanalization has been accomplished during which time these interventions could be effectively implemented.

We suggest that future animal studies of cardioprotective interventions be conducted on a background of a $P2Y_{12}$ inhibitor to provide a more clinically relevant model. Unless an agent can provide additive protection in that model, it would be of little clinical value. Although we have tested IPC and IPoC combined with the $P2Y_{12}$ inhibitor cangrelor and have found no additional protection, it is unknown whether interventions such as remote conditioning (Lim and Hausenloy, 2012) or promoters of autophagy (Sala-Mercado *et al.*, 2010) whose mechanisms are less well understood might have additive effects.

Concluding remarks

Our 43-year journey has brought us to this point where we have a good understanding of the type of intervention that must be introduced to spare ischaemic myocardium. Now that conditioning's protection is being applied to patients routinely with the antiplatelet drugs, we should look elsewhere for the next generation of cardioprotective drugs. We propose that an intervention not based on the signalling of IPC or IPoC would be most likely to add protection to that already resulting from treatment with standard antiplatelet agents. Thus, IPoC or even cyclosporin which prevents formation of mPTP would be expected to have only small, if any, effect in this setting. Infarct size reduction clearly reduces mortality and morbidity in AMI as clinical trials with reperfusion therapy and $P2Y_{12}$ inhibitors have proven. Yet further protection against infarction is still indicated as AMI continues to be a deadly and debilitating disease. The preclinical studies completed by many investigators have established the foundation for cardioprotective strategies and we must continue to search for new strategies to preserve myocardium from a transient ischaemic insult. In the past, the improvements in outcome in AMI have been incremental rather than revolutionary. By using relevant animal models, we can hopefully identify candidates for future clinical testing and someday make myocardial infarction a mere historical medical footnote.

Conflicts of interest

None.

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