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Review

New directions for protecting the heart against ischaemia–reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway

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Abstract

Reperfusion is a pre-requisite to salvaging viable myocardium, following an acute myocardial infarction. Reperfusion of ischaemic myocardium, however, is not without risk, as the act of reperfusion itself can paradoxically result in myocyte death: a phenomenon termed lethal reperfusion-induced injury. Therapeutic strategies that target and attenuate reperfusion-induced cell death may provide novel pharmacological agents, which can be used as an adjunct to current reperfusion therapy, to limit myocardial infarction. Recent evidence has implicated apoptotic cell death during the phase of reperfusion as an important contributor to lethal reperfusion-induced injury. Targeting anti-apoptotic mechanisms of cellular protection at the time of reperfusion may therefore offer a potential approach to attenuating reperfusion-induced cell death. In this regard, ischaemia–reperfusion has been shown to activate the anti-apoptotic pro-survival kinase signalling cascades, phosphatidylinositol-3-OH kinase (PI3K)–Akt and p42/p44 extra-cellular signal-regulated kinases (Erk 1/2), both of which have been implicated in cellular survival. Activating these pro-survival kinase cascades at the time of reperfusion has been demonstrated to confer protection against reperfusion-induced injury. We and others have shown that insulin, insulin-like growth factor-1 (IGF-1), transforming growth factor-β1 (TGF-β1), cardiotrophin-1 (CT-1), urocortin, atorvastatin and bradykinin protect the heart, by activating the PI3K–Akt and/or Erk 1/2 kinase cascades, when given at the commencement of reperfusion, following a lethal ischaemic insult. Pharmacological manipulation and up-regulation of these pro-survival kinase cascades, which we refer to as the Reperfusion Injury Salvage Kinase (RISK) pathway, as an adjunct to reperfusion may therefore protect the myocardium from lethal reperfusion-induced cell death and provide a novel strategy to salvaging viable myocardium and limiting infarct size.

Keywords: Reperfusion injury; Protein kinases; Growth factors

1. Reperfusion-induced injury

Coronary heart disease represents a global burden on healthcare resources and is poised to become the leading cause of morbidity and mortality in the world by 2020, according to the World Health Organisation [1]. Novel therapeutic strategies are urgently required to tackle the consequences of coronary artery disease in order to reduce the global impact of this disease on society. Following an acute myocardial infarction, re-establishing coronary blood flow with the rapid use of reperfusion strategies such as thrombolysis or primary angioplasty is essential to salvage viable myocardium. However, reperfusion of ischaemic myocardium carries with it an inherent risk, in that paradoxically, the process of reperfusion can itself result in myocyte death—a phenomenon termed lethal reperfusion-induced injury [2].

The existence of lethal reperfusion injury as a separate entity is controversial, with some commentators suggesting that reperfusion exacerbates the cellular injury sustained during the ischaemic period [3]. Studies have demonstrated that reperfusion can exacerbate the necrotic component of cell death as evidenced by an extension in infarct size, following a fixed period of ischemia [4,5]. Other studies, on the other hand, indicate that the oxidative stress and abrupt metabolic changes that accompany reperfusion can initiate

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cellular injury in the absence of ischaemia [6,7]. The most convincing means of demonstrating the existence of lethal reperfusion injury is to show that myocyte death can be modified by interventions administered *at the time of reperfusion*.

In this article, we review a therapeutic strategy that has been demonstrated to attenuate myocardial injury when applied during the first few minutes of reperfusion. This approach involves the activation of specific signalling kinase cascades, which, in turn, protect the heart from reperfusion-induced cell death by recruiting innate cellular anti-apoptotic pathways of survival.

2. The contribution of apoptotic cell death to ischaemia–reperfusion injury

Recent advances in our understanding of cell death during ischaemia–reperfusion implicate two forms of cell death in the pathology of a myocardial infarction, namely necrosis and apoptosis [8]. Apoptosis is a regulated, energy-dependent process that results in chromatin condensation, DNA fragmentation and apoptotic body formation, preserved cell membrane integrity, without an associated inflammatory response [9]. In contrast, necrosis is characterised by membrane disruption, massive cell swelling, cell lysis and fragmentation, with an associated acute inflammatory response. The exact contribution of these two forms of cell death in the setting of ischaemia–reperfusion injury is unclear, as are the factors that determine whether the apoptotic or necrotic death pathway is recruited.

Apoptotic cell death in the rat heart has been demonstrated to be induced by a prolonged episode of ischaemia alone, in the absence of reperfusion [10,11]. Some studies have suggested that reperfusion accelerates the apoptotic death process initiated during ischaemia [10,12–14]. In contrast, several studies suggest that the apoptotic component of cell death is triggered at the time of reperfusion and does not manifest during the ischaemic period [15]. Therefore, the evidence suggests that the apoptotic component of cell death is either triggered or accelerated during the reperfusion phase. The fact that apoptosis is an energy-dependent process and ATP levels are depleted during ischaemia and replenished on reperfusion may explain why the apoptotic component of cell death is associated with reperfusion [16].

The relationship between apoptotic and necrotic cell death in the setting of ischemia–reperfusion injury is also unresolved, with some commentators suggesting that there may be considerable overlap in terms of early signalling events between these two pathways, an observation that may be useful in terms of developing therapeutic targets for clinical use. Zhao et al. [17] have characterised, using a canine model of ischaemia–reperfusion injury, the contribution of necrotic and apoptotic cell death. They demonstrated that these two forms of cell death occur simultaneously during the reperfusion phase, with necrotic cell death peaking after 24 h of reperfusion, and apoptotic cell death increasing up to 72 h of reperfusion. Other studies have demonstrated that the pharmacological inhibition of the apoptotic signalling cascade during the reperfusion phase is able to attenuate both the apoptotic and necrotic components of cell death [18–21], suggesting that the apoptotic death process can evolve into necrotic cell death. As well as the apoptotic component of cell death contributing to the extension of infarct size during reperfusion, a study by Zhao et al. [21] demonstrated that pharmacologically inhibiting the reperfusion-induced apoptotic component of cell death also resulted in improved contractile function of ischaemic canine hearts. These studies suggest that targeting the reperfusion-induced apoptotic component of cell death can impact on both the apoptotic and necrotic components of cell death, the consequences of which are a reduction in infarct size and improved contractile function.

However, although it is fair to state that the majority of evidence supports the role of apoptosis in ischaemia–reperfusion injury, because of unresolved issues surrounding the contribution of apoptosis to the pathophysiology of ischaemia–reperfusion injury, several authors still question the significance of apoptosis in this setting [22]. For example, methodological issues concerning the detection of apoptosis in the heart were questioned in a study by Ohno et al. [23], in which immunogold electron microscopy and in situ nick end labelling, revealed that coronary artery occlusion in the rabbit resulted in detection of necrotic and not apoptotic cell death [24], and Taimor et al. [25] could only demonstrate the induction of necrosis but not apoptosis in isolated rat myocytes subjected to hypoxia–reoxygenation.

3. Targeting the apoptotic component of reperfusion-induced cell death by activating the pro-survival kinase cascades

In order to target the apoptotic component of reperfusion-induced cell death, the activation of existing innate cellular anti-apoptotic pathways of survival, may afford an opportunity for protecting the heart against lethal reperfusion-induced injury. Ischaemia–reperfusion has been shown to activate the pro-survival kinase signalling cascades, phosphatidylinositol-3-OH kinase (PI3K)–Akt and p42/p44 extra-cellular signal-regulated kinases (Erk 1/2), both of which have been implicated in cellular survival, through their recruitment of anti-apoptotic pathways of protection [26]. There are of course several other kinases that have been implicated in the setting of ischaemia–reperfusion injury through their effects on apoptotic cell death, such as p38 and JNK MAPK, PKA, Rho kinase and JAK-STAT. These, however, are not covered in this article as they are beyond the scope of this review.

3.1. The pro-survival PI3K–Akt and MEK 1/2–Erk 1/2 signalling cascades

The PI3K–Akt signalling cascade is activated in response to the activation of a wide range of receptors, including those for growth factors and G-protein-coupled receptors [26]. The PI3K–Akt pathway participates in numerous cellular processes by phosphorylating a diverse array of substrates, including glycogen synthase kinase-3 (glycogen and protein metabolism), apoptotic proteins (BAD, BAX, BIM, p53 and caspases), GLUT4 vesicles (glucose metabolism), transcription factors (IKK-α and Forkhead proteins), p70S6K, eNOS and PKC [26]. Signalling through PI3 kinase has been demonstrated to confer protection against ischaemia–reperfusion injury [27,28], through its activation of the serine–threonine kinase, Akt [29].

The Erk 1/2 or p42/p44 signalling cascade is a member of the mitogen-activated protein kinases (MAPKs), a family of serine–threonine kinases concerned with the regulation of cell proliferation, differentiation and survival, which is activated in response to the occupation of tyrosine kinase and G-protein-coupled receptors [30]. The Erk 1/2 cascade, when activated in the setting of ischaemia–reperfusion, can mediate cellular protection [31,32].

The mechanism through which the recruitment of these pro-survival kinase pathways mediates cellular protection is not certain, but cellular survival has been attributed in part to their ability to phosphorylate and inactivate a diverse array of pro-apoptotic proteins.

3.1.1. Phosphorylation and inactivation of the pro-apoptotic proteins bad, BAX, BIM and p53

Activation of the PI3K–Akt or the MEK 1/2–Erk 1/2 cascades phosphorylate the pro-apoptotic protein BAD, either directly [33] or indirectly via the recruitment of distal signalling moieties such as the 70-kDA ribosomal protein S6 kinase (p70S6K) [34] or the p90 ribosomal S6 kinase (p90RSK) [35]. Phosphorylation of BAD results in its binding to 14-3-3, which sequesters it from its mitochondrial target, thereby preventing apoptosis [36].

In response to an apoptotic stimulus, the pro-apoptotic protein, Bax, undergoes a conformational change that allows it to translocate to the mitochondria [37,38], where it induces mitochondrial cytochrome *c* release by either forming a pore in the outer mitochondrial membrane itself or by interacting with and opening the mitochondrial permeability transition pore (mPTP) [39]. Activation of either the PI3K–Akt or the Erk 1/2 pathway inhibits the conformational change in BAX required for its translocation to the mitochondria, therefore preventing apoptosis [37,38,40].

Withdrawal of survival factors results in the expression de novo of the pro-apoptotic protein, BIM [40]. Weston et al. [40] demonstrated that the inhibition of either the PI3K–Akt or the Erk 1/2 pathways resulted in an increase in BIM expression, implying that these pathways may exert an inhibitory influence on BIM.

By phosphorylating Mdm2, activation of the PI3K–Akt pathway targets the pro-apoptotic protein, p53, for degradation, thereby preventing apoptosis [41].

3.1.2. Inhibiting mitochondrial cytochrome c release and phosphorylating and inactivating caspases, the executors of apoptotic cell death

Kennedy et al. [42] found that Akt was able to inhibit mitochondrial cytochrome *c* release and maintain mitochondrial membrane potential, independent of BAD. One potential route for mitochondrial cytochrome *c* release into the cytosol is through the opening of the mPTP [43]. Based on this observation, it would be interesting to postulate that Akt may actually suppress cytochrome *c*-induced apoptosis by inhibiting opening of the mPTP, thereby retaining cytochrome *c* within the mitochondrial intermembranous space (see Section 5).

Erhardt et al. [44] demonstrated that over-expressing B-raf in a fibroblast cell line (which results in activation of Erk 1/2), rendered cells resistant to cytochrome *c*-induced apoptosis. Given that mitochondrial cytochrome *c* is required to activate caspases, the findings of this study suggest that Erk 1/2 activation is able to inhibit cytochrome *c*-induced caspase activation. A potential explanation for this may be that up-regulation of the Erk 1/2 cascade inactivates one component of the caspase cascade, a proposition which is supported by the finding that Erk 1/2 kinase activation has been shown to inhibit apoptosis, by inhibiting caspase 3 activation, in haematopoietic cells [45]. Furthermore, by phosphorylating and inactivating pro-caspase 9, Akt activation can suppress the mitochondrial apoptotic death pathway [46].

In addition to influencing components of the apoptotic signalling pathway, activation of these kinase cascades may also induce cellular protection through the phosphorylation and activation of non-apoptotic proteins.

3.1.3. Phosphorylation and activation of endothelial nitric oxide synthase (eNOS)

Akt has been shown to phosphorylate eNOS, producing nitric oxide which has been implicated in cellular protection [47]. Nitric oxide, in turn, has been shown to inhibit opening of the mPTP [48]. Based on this finding, we postulate that activating the PI3–Akt pathway during the first few minutes of reperfusion, protects the myocardium by inhibiting the opening of the mPTP, which normally occurs at reperfusion (see Section 5).

3.1.4. Activation of protein kinase C (PKC)

Signalling through the PI3K–Akt kinase pathway has been demonstrated to activate PKC [49]. This protein kinase has been shown to mediate the cellular protection associated with the phenomenon of ischaemic preconditioning (IPC) [50], in which one or more transient sub-lethal episodes of ischaemia render the myocardium resistant to a subsequent more prolonged episode of lethal ischaemia [51]. A further potential anti-apoptotic mechanism afforded by Akt activa-

tion, which is also dependent on PKC, is the activation of mitochondrial Raf-1 [52], which has been shown to phosphorylate and inactivate the pro-apoptotic factor, BAD [53].

3.1.5. Phosphorylation of factors concerned with the regulation of gene expression

Akt phosphorylates and activates IKK-α, which leads to the activation and translocation of NF-κB to the nucleus, where it acts as a transcription factor for a variety of survival pathways [54]. The contribution of NF-κB to apoptotic cell death during the reperfusion phase is inconclusive, with studies showing it to be anti-apoptotic [55], while others suggesting that it has a pro-apoptotic action [56].

Akt has also been demonstrated to phosphorylate and inhibit the Forkhead transcription factor FKHRL1 by sequestering it in the cytosol in association with 14-3-3 protein and preventing FKHRL1-mediated transcription of death-inducing genes such as Fas ligands [57].

Erk 1/2-mediated phosphorylation of p90RSK has been linked to the regulation of the gene expression of cAMP-response element-binding (CREB) protein, which transcribes genes concerned with cellular survival [58].

4. Protecting the heart against ischaemia injury by activating the pro-survival kinase cascades at the time of reperfusion: the Reperfusion Injury Salvage Kinase (RISK) pathway

Activation of the pro-survival kinase cascades, during the first few minutes of reperfusion, following a lethal ischaemic insult, has been hypothesised to attenuate reperfusion-induced cell death via the various anti-apoptotic mechanisms listed previously [59]. As activation of these pro-survival kinases at the time of reperfusion appears to be sufficient to induce a cardio-protective response, we use the term RISK pathway to represent the PI3K–Akt and Erk 1/2 pro-survival kinase cascades that have been implicated in protecting the heart against cell death during the reperfusion phase. Therefore, the ability to manipulate and up-regulate the RISK pathway, during the early reperfusion phase may provide a potential approach to limiting reperfusion-induced cell death.

In this regard, a number of growth factors and other agents have been shown to induce cardio-protection in the setting of ischaemia–reperfusion injury. This article will focus on those that have been shown to protect the heart when given *during the early reperfusion phase*, and whose mechanism of protection has been linked to activation of the pro-survival kinase cascades.

4.1. Insulin protects at reperfusion by activating the PI3K–Akt kinase pathway

We have recently reviewed the potential mechanisms associated with insulin-mediated cardio-protection at the time of reperfusion and our studies indicate the activation of the pro-survival PI3K–Akt cascade during the first few minutes of reperfusion as being essential for protection [60]. Jonassen et al. [61] demonstrated a reduction in infarct size associated with the administration of glucose–insulin–potassium (GIK) at the time of reperfusion, and in studies using cardiomyocytes subjected to hypoxia, our group demonstrated that insulin given, at the time of reoxygenation, attenuated the apoptotic and necrotic components of cell death [62]. Insulin-mediated cardio-protection was also shown to correlate with phosphorylation of Akt and BAD, with insulin inducing phosphorylation of Akt and BAD to a level greater than that observed in control hearts (see Fig. 1A) [63]. In the isolated perfused rat heart, early reperfusion of insulin was shown to limit infarct size, an effect that was abrogated in the presence of both wortmannin (the PI3K inhibitor) or rapamycin (the mTOR-p70S6K inhibitor) (see Fig. 1B) [63]. In this study, it was also shown that 15 min administration of insulin was sufficient to induce protection. Furthermore, early reperfusion of insulin was essential, as delaying its administration to 15 min after the onset of reperfusion, was not associated with protection. The fact that insulin has to be present in the first few minutes of reperfusion to induce protection lends support to the hypothesis that insulin protects at reperfusion by inhibiting opening of the mPTP, as opening of the latter has been shown mediate cell death in the first few minutes of reperfusion (see Section 5). A study by Gao et al. [64] has also implicated eNOS, another downstream target of Akt

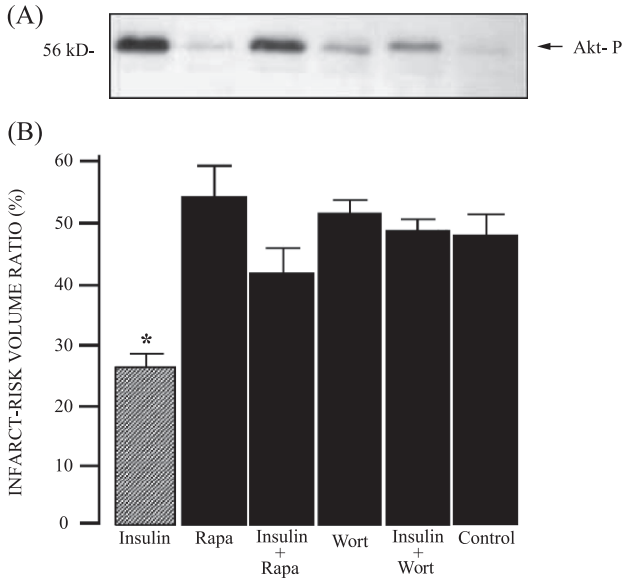

Fig. 1. (A) Western blots showing Akt phosphorylation at 10 min post-ischaemic reperfusion in isolated perfused rat hearts treated with insulin, wortmannin (the PI3K inhibitor) and rapamycin (the mTOR-p70S6K inhibitor) for the first 15 min of reperfusion. $N=3$ per group. (B) Graph showing the infarct-risk volume ratios in isolated perfused rat hearts treated with insulin, wortmannin (Wort) and rapamycin (Rapa) for the first 15 min of reperfusion. $N \geq 6$ per group. $^{*}P<0.01$. Data taken from a study by Jonassen et al. [63].

phosphorylation, in insulin-mediated cardio-protection at reperfusion.

4.2. Insulin-like growth factor-1 (IGF-1) protects at reperfusion by activating both the PI3K–Akt and Erk 1/2 cascades

IGF-1 is a serum factor implicated in cellular survival and growth that has been shown to reduce apoptosis in a wide variety of cells in response to a diverse array of stimuli. IGF-1 has been demonstrated to protect the heart against ischaemia–reperfusion injury, by attenuating both apoptotic and necrotic cell death [65,66] in a manner that was dependent on the PI3K–Akt and Erk 1/2 signalling cascades [28,67].

In the isolated perfused rat heart, the administration of IGF-1 at reperfusion induced cardio-protection that was sensitive to wortmannin [68]. Studies in transgenic mice over-expressing IGF-1 were found to have a higher basal activation of Akt and, at reperfusion, the level of Akt activation was amplified even further [66]. In these mice, the increased levels of Akt activation were demonstrated to correlate with protection and were also shown to be wortmannin-sensitive [66]. Downstream of the these kinase cascades, BAD [69], Bax, caspase 3 [70] and p70S6K have been implicated in IGF-1-induced cellular protection.

4.3. Transforming growth factor-β1 (TGF-β1) protects at reperfusion by recruiting the Erk 1/2 signalling cascade

TGF-β1 is a cytokine that regulates cell growth and differentiation and modulates apoptosis in many cell types. The cardio-protective properties of TGF-β1 were first investigated in the early 1990s by Lefer et al. [71] who demonstrated protection against ischaemia–reperfusion injury in the rat heart ex vivo and in vivo.

Our group were the first to study the effect of TGF-β1 when given at the point of reoxygenation/reperfusion in rat myocytes and in the isolated perfused rat heart, respectively [72]. In this study, TGF-β1 given during the reoxygenation phase following an episode of lethal hypoxia was shown to be protective, demonstrated by attenuated trypan blue uptake and a reduction in the apoptotic component of cell death (assessed by a reduction in TUNEL and annexin V-positive cells), and in the isolated perfused rat heart, treatment with TGF-β1 for the first 15 min of reperfusion, following an episode of lethal ischaemia, was associated with a significant reduction in infarct size. In both these cases, TGF-β1-induced protection was demonstrated to be dependent on the Erk 1/2 cascade [72].

4.4. Cardiotrophin-1 (CT-1) protects at reperfusion by recruiting the PI3K–Akt and Erk 1/2 signalling cascades

CT-1 is a member of the interleukin 6 (IL-6) family of cytokines, which was originally isolated for its ability to induce a hypertrophic response in isolated cardio-myocytes [73], by signalling through the gp130 trans-membrane protein [74]. In addition to its hypertrophic-inducing action, CT-1 has since been shown to be cardio-protective, exerting an anti-apoptotic effect in response to serum withdrawal in cardiac myocytes via an Erk 1/2-dependent pathway [75]. Using the isolated rat myocyte and the intact rat heart models, our group demonstrated that, when given at point of reoxygenation/reperfusion, CT-1 induced cardio-protection via activation of the MEK 1/2–Erk 1/2 [76,77] and the PI3K–Akt pathways [78].

4.5. Urocortin protects the heart at reperfusion by recruiting the PI3K–Akt and Erk 1/2 signalling cascades

Urocortin, a peptide related to hypothalamic corticotrophin releasing factor, is released by myocytes in response to stressful stimuli such as ischaemia [79]. Using neonatal rat myocytes, Brar et al. [80] demonstrated that after a prolonged episode of hypoxia, the presence of urocortin, at the time of reoxygenation, prevented cell death, by an anti-apoptotic action (represented as a reduction in annexin V and TUNEL staining). We went on to demonstrate, in the isolated perfused rat heart and in vivo rat heart subjected to prolonged ischaemia, that urocortin given at the time of reperfusion reduces infarct size [80,81]. In these studies, the potential mechanism associated with this protection was examined in detail.

In this regard, our group have demonstrated that the cardio-protection observed with giving urocortin at the point of reperfusion was mediated via the Erk 1/2 cascade [80,81]. Of importance is the fact that in these studies we demonstrated that urocortin caused an increase in Erk 1/2 phosphorylation over and above the level observed by reperfusion alone (see Fig. 2A) [80,81]. In addition, the infarct-limiting effect associated with urocortin was abrogated in the presence of the MEK 1/2 inhibitor PD 098059, and this effect was accompanied by a reduction in urocortin-induced Erk 1/2 phosphorylation (Fig. 2A and B) [81].

Furthermore, activation of the PI3 kinase–Akt cascade has also been implicated in urocortin-mediated protection [82]. In this study, urocortin-mediated protection against hypoxia–reoxygenation injury was abrogated in rat myocytes treated with the specific PI3K inhibitors, wortmannin or LY294002. In addition, urocortin-induced protection in the same setting was abrogated in neonatal rat myocytes possessing a dominant negative mutation of PI3K–Akt [82].

4.6. Fibroblast growth factor protects at reperfusion via recruitment of the Erk 1/2 signalling cascade

Fibroblast growth factor-2 (also known as basic fibroblast growth factor, FGF) is a polypeptide growth factor, which has been shown to modulate cell proliferation,

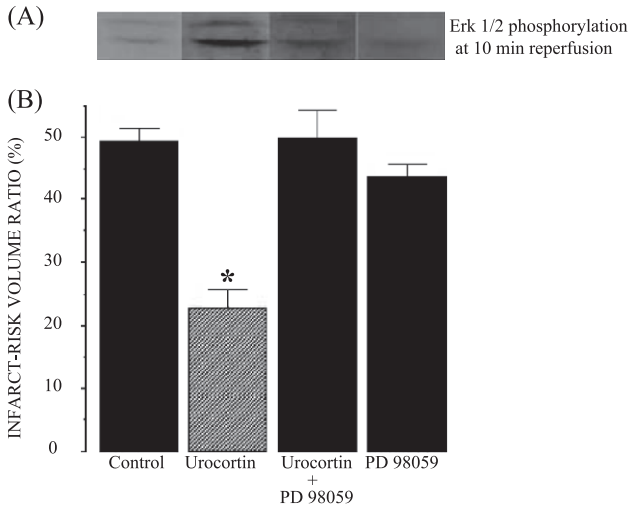

Fig. 2. (A) Western blots showing p42/p44 MAP kinase (Erk 1/2) phosphorylation at 10 min post-ischaemic reperfusion in isolated perfused rat hearts treated with urocortin, and PD 98059 (the MEK 1/2 inhibitor) for the first 15 minutes of reperfusion. $N=3$ per group. (B) Graph showing the infarct-risk volume ratios in isolated perfused rat hearts treated with urocortin and PD 98059 for the first 15 minutes of reperfusion. $N \geq 6$ per group. $^*P<0.01$. Data taken from a study by Schulman et al. [81].

survival and apoptosis [83]. FGF has been demonstrated to induce cardio-protection, when administered during the reperfusion phase in a rat model of myocardial infarction in a nitric oxide dependent manner [84,85] and has been associated with attenuation of apoptotic cell death [86]. Studies have implicated PKC and the Erk 1/2 pathway in FGF-mediated cardio-protection [87,88]. Jiang et al. [89] demonstrated cardio-protection in the isolated perfused rat heart when FGF was given during the first 12 min of reperfusion, following 30 min of global ischaemia, and protection was shown to be PKC-dependent. The fact that FGF-mediated cardio-protection has been shown to be dependent on PKC and possibly K_{ATP} channels [90] suggests that FGF may protect via a similar mechanism to ischaemic preconditioning [50].

4.7. Other cardio-protective growth factors in which the pro-survival kinases have been implicated

Several other growth factors including vascular endothelial growth factor (VEGF) [91] and hepatic growth factor (HGF) [92,93] have been shown to cardio-protect against ischaemia–reperfusion injury but have not been examined at the time of reperfusion. HGF was demonstrated to induce activation of Erk 1/2 kinase, but the contribution of this kinase to cellular protection was not examined in this study [93].

Other growth factors such as epidermal growth factor (EGF) [94], nerve growth factor (NGF) [95] and platelet-derived growth factor (PDGF) [95] have been shown to be protective but have not yet been investigated in cardiac tissue. We can postulate that if these growth factors activate the pro-survival kinases cascades of the RISK pathway, in myocardial tissue, one might expect them to also protect the heart against reperfusion injury.

4.8. The hydroxyl-3-methylglutaryl (HMG)-co-enzyme A (CoA) reductase inhibitor, atorvastatin protects at reperfusion by recruiting the PI3K–Akt signalling cascade

The HMG-CoA reductase inhibitors or "statins" have been shown to be cardio-protective in large-scale primary [96] and secondary prevention studies [97]. In addition to its cholesterol-lowering effect, this class of drugs has been associated with many pleiotropic effects, many of which mediate their cardio-protective effect [98]. HMG-CoA reductase inhibitors have also been shown to up-regulate the PI3K–Akt kinase cascade in endothelial cell lines [99].

As such, we hypothesised that statins by virtue of their ability to up-regulate PI3K–Akt should also be able to protect the myocardium when given at the moment of reperfusion. In this regard, we have recently shown, using the isolated perfused mouse heart model, that the HMG-CoA reductase inhibitor, atorvastatin, administered during the early reperfusion phase, limited infarct size via recruitment of the PI3K–Akt kinase pathway [100]. The presence of wortmannin (the PI3K inhibitor) during the reperfusion phase abrogated the atorvastatin-induced reduction in infarct size with a concomitant attenuation of atorvastatin-induced Akt activation. Furthermore, the downstream activation of eNOS was also implicated in atorvastatin-induced cardio-protection at reperfusion, based on the finding that atorvastatin provided no cardio-protection in mice with a targeted deletion of the eNOS gene.

This was the first study to demonstrate non-receptor mediated activation of the PI3K–Akt kinase pathway mediating cardio-protection at the time of reperfusion [100].

4.9. The G-protein receptor ligand, bradykinin, protects at reperfusion by recruiting the PI3K–Akt signalling cascade

Treatment with angiotensin-converting enzyme inhibitors (ACE-I) has been linked to cardio-protection in the setting of ischaemia–reperfusion injury [101]. Studies have demonstrated that ACE-I-induced cardio-protection is mediated by bradykinin (acting at the B2 receptor) and is dependent on nitric oxide [102]. Interestingly, it has been shown that Gq-protein receptors, such as the bradykinin B2 receptor signal through the PI3K pathway in the guinea pig heart [103].

Based upon this, we demonstrated for the first time a link between G-protein-coupled receptor activation at reperfusion, using bradykinin, and cardio-protection via recruitment of the PI3K–Akt pathway [104]. Using the isolated perfused mouse heart model, we found that bradykinin administered during the first few minutes of reperfusion, limited infarct size via recruitment of the PI3K–Akt kinase pathway [100]. The presence of wortmannin (the PI3K

inhibitor) during the reperfusion phase abrogated the bradykinin-induced reduction infarct size with a concomitant attenuation of bradykinin-induced Akt activation. Furthermore, bradykinin-induced cardio-protection was shown to be eNOS dependent, as bradykinin provided no cardio-protection in transgenic eNOS knockout mice.

Interestingly, a recent study in bovine aortic endothelial cells has shown that bradykinin can activate Erk 1/2 and eNOS activation, independent of the PI3–Akt pathway [105]. This suggests that the Erk 1/2 kinase pathway may also contribute to bradykinin-mediated cardio-protection at reperfusion.

This study was the first to demonstrate G-protein-coupled receptor activation at reperfusion mediating cardio-protection via activation of the PI3K–Akt component of the RISK pathway [104]. It would be interesting and important to determine whether protection at reperfusion can be induced by other G-protein-coupled receptor ligands. In this regard, we have examined activation of the G-protein-linked adenosine receptor.

4.10. G-protein-coupled receptor activation by certain adenosine agonists mediate protection against reperfusion injury via recruitment of the Erk 1/2 signalling cascade

Xu et al. [106] demonstrated that AMP579 (an adenosine A1/A2a receptor agonist) given during the reperfusion phase limited infarct size using the in vivo rabbit heart model of ischaemia–reperfusion injury. Interestingly, activation of the adenosine A1, A2 and A3 receptor has been associated with phosphorylation of the Erk 1/2 kinase cascade in Chinese Hamster ovary cells [107]. Using the in vivo rabbit heart model of ischaemia–reperfusion injury, we found that AMP579-induced protection at reperfusion, was abrogated in the presence of PD 098059 (the MEK 1/2 inhibitor) [108].

We have recently examined the role of the adenosine A3 receptor in cardio-protection at reperfusion. In the adult rat myocyte subjected to hypoxia–reoxygenation, we found that administering the A3 receptor agonist, 2-Cl-IB-MECA, at time of reoxygenation attenuated both the apoptotic and necrotic components of cell death [109]. In the isolated perfused rat heart subjected to ischaemia–reperfusion, we demonstrated that the presence of 2-Cl-IB-MECA, during the first few minutes of reperfusion limited infarct size [109]. Given that, activation of the adenosine A3 receptor has been linked to Erk 1/2 kinase activation [107], we can postulate that this component of the RISK pathway may be implicated in A3 receptor-mediated cardio-protection at reperfusion.

4.11. Ischaemic preconditioning protects against ischaemia–reperfusion injury by recruiting the PI3K–Akt and Erk 1/2 signalling cascades at the time of reperfusion

IPC, which describes the phenomenon in which transient non-lethal episodes of myocardial ischaemia protect the myocardium against a subsequent prolonged ischaemic episode, exerts profound protection against ischaemia–reperfusion injury [51]. Despite intensive investigation, the actual mechanism of IPC-induced cardio-protection remains uncertain [50]. Activation of the PI3K–Akt and Erk 1/2 signalling cascades *prior* to the lethal ischaemic insult has been shown to mediate IPC-induced cardio-protection [110–112]. Given the mounting evidence supporting the role of pro-survival kinases inducing protection at reperfusion, we recently postulated that IPC may also mediate cardio-protection by up-regulating the pro-survival PI3K–Akt and Erk 1/2 kinase signalling cascades, *at the time of reperfusion*, following the sustained ischaemic period. In this regard, we have undertaken preliminary experiments using the isolated perfused rat heart model of infarction, and demonstrate that IPC results in phosphorylation of both Akt and Erk 1/2 during the first few minutes of reperfusion [113]. Interestingly, we also found that phosphorylation of these kinases was essential to mediate IPC-induced protection, as the presence of either PD098059 (the MEK 1/2 inhibitor) or LY294002 (the PI3K inhibitor) for the first 15 min of reperfusion abrogated the IPC-induced infarct-limiting effect and also abolished the IPC-induced phosphorylation of Akt and Erk 1/2, respectively. This preliminary data suggests that up-regulation of the pro-survival PI3K–Akt and Erk 1/2 kinase signalling cascades, which comprise the RISK pathway, during the first few minutes of reperfusion, mediates the protection associated with ischaemic preconditioning. Further studies are required to confirm these findings.

5. Protection at reperfusion by activating the RISK pathway: the potential downstream mediators and end-effector of this protection

The activation of the pro-survival PI3K–Akt and MEK 1/2–Erk 1/2 cascades at the time of reperfusion, by ligands to growth factor or G-protein-coupled receptors, protects the heart against lethal reperfusion injury. From the available evidence, it appears that BAD, BAX, p70S6K and eNOS appear to be the downstream components responsible for mediating the protection associated with the activation of these kinase cascades at the time of reperfusion. Further work is required to ascertain whether these components actually constitute a common pathway for all agents which protect the heart when given at the time of reperfusion.

Many of the anti-apoptotic pathways activated by the pro-survival kinase cascades converge on the mitochondria, which should come as no surprise given that the latter are believed to play a fundamental role in the apoptotic death machinery [114]. Within mitochondria, it is the mPTP that appears to occupy a fundamental role in determining cellular survival in the setting of ischaemia–reperfusion injury [115]. The mPTP is a non-specific large conductance pore of the inner mitochondrial membrane whose opening may

determine whether cell death occurs by apoptosis or necrosis. Opening of the mPTP has been shown to take place in the first few minutes of reperfusion [116], and we and others have demonstrated that pharmacologically inhibiting its opening at this time is cardio-protective [117–119]. Therefore, inhibition of mPTP opening may be mediated as a consequence of kinase activation by removing the pro-apoptotic proteins, BAD, BAX and p53 from their mitochondrial site of action (see Section 3.1.1 and Fig. 4). In addition, the AKT-induced activation of eNOS and the resultant nitric oxide release may also inhibit mPTP opening, in this setting (see Section 3.1.3 and Fig. 4).

We postulate and are currently investigating whether the protection associated with activation of pro-survival kinase cascades PI3K–Akt and Erk 1/2 at the time of reperfusion is mediated by inhibition of mPTP opening during this crucial time. For further details of the potential importance of the mPTP in the setting of ischaemia–reperfusion injury, the reader is directed to the review by Halestrap et al. in this issue.

6. Agents that precondition versus those that protect when given at reperfusion: Do the PI3K–Akt and Erk 1/2 cascades constitute a common pathway for their cardio-protection?

This article has focused on the role of the pro-survival PI3K–Akt and Erk 1/2 kinase cascades as mediating protection at the time of reperfusion. However, it is interesting to note that the same kinase cascades have also been implicated in mediating the protection associated with the phenomenon of IPC [110–112]. In this scenario, activation of the kinase cascades occurs prior to the ischaemic insult and acts as a preconditioning trigger and/or mediator for cardio-protection [50].

In the light of this evidence, we propose that these kinase cascades may constitute a common pathway of cardio-protection, mediating the protection associated with both IPC and the RISK pathway. Evidence in support of this proposition is provided by the fact that agents which precondition, such as bradykinin and AMP579 [120,121], also induce protection when given at reperfusion [104,108]. Conversely, agents that have been demonstrated to protect at reperfusion by activating the RISK pathway, such as insulin, urocortin and CT-1 have also been shown to precondition the myocardium [122–124].

The evidence would tend to suggest that the pro-survival kinase cascades may therefore constitute a common pathway, mediating the cardio-protection induced by IPC on the one hand, as well as protecting the myocardium through their recruitment at the time of reperfusion on the other hand. However, the only direct evidence for this rests with insulin-induced cardio-protection, and, therefore, more research is needed to elucidate whether the pro-survival kinase cascades actually constitute the common pathway for cardio-protection in these two settings.

7. The PI3K–Akt and Erk 1/2 kinase cascades constitute a universal pro-survival signalling pathway mediating myocardial protection at reperfusion—clinical implications

Activation of the PI3K–Akt and Erk 1/2 kinase cascades appear to constitute a universal pro-survival kinase cascade mediating cardio-protection at reperfusion. Many of the growth factors and agents that initiate cardio-protection when given during the reperfusion phase appear to activate either one or both of these pro-survival kinase cascades that comprise the RISK pathway (see Figs. 3 and 4). Protection mediated by the activation of the RISK pathway, appears to be executed via anti-apoptotic pathways that induce cellular protection (Fig. 4). We would propose, therefore, that therapeutic interventions which target and activate the RISK pathway during the reperfusion phase can be used as an adjunct to current reperfusion therapy, and may provide an approach to salvaging viable myocardium and limiting infarct size in patients presenting with an acute myocardial infarction. Already, a large randomised control clinical study (named GIK II) is underway, examining the benefits of glucose insulin therapy (GIK) given at the time of reperfusion in patients presenting with an acute myocardial infarction [125].

An alternative strategy would be to investigate whether drugs that have been clearly demonstrated to be cardio-protective and are routinely used to treat chronic ischaemic heart disease provide any benefit if given at the time of reperfusion. For example, we have shown that administering either a statin or bradykinin (which would be expected to be raised in response to ACE-inhibitor therapy) at the time of

Agents which protect against reperfusion injury by up-regulating the RISK pathway	Receptor	RISK Pathway	
		P13K-Akt	Erk 1/2
Adenosine agonists (A1/A2a/A3 receptor)	G-protein coupled		✓
Bradykinin	G-protein coupled	✓	✓
Cardiotrophin-1 (CT-1)	gp130	✓	✓
Fibroblast growth factor (FGF)	T.K		✓
Insulin	T.K	✓	✓
Insulin-Like Growth Factor-1 (IGF-1)	T.K	✓	✓
Transforming growth factor-β1 (TGF-β1)	Ser Thr kinase		✓
Urocortin	T.K	✓	✓
Atorvastatin	n/a	✓	

Fig. 3. Table showing the list of agents that have been demonstrated to protect the heart at reperfusion, following an episode of ischaemia, showing the receptor and pro-survival kinase cascades implicated in their protection (RISK pathway: Reperfusion Injury Salvage Kinases pathway; T.K: tyrosine kinase).

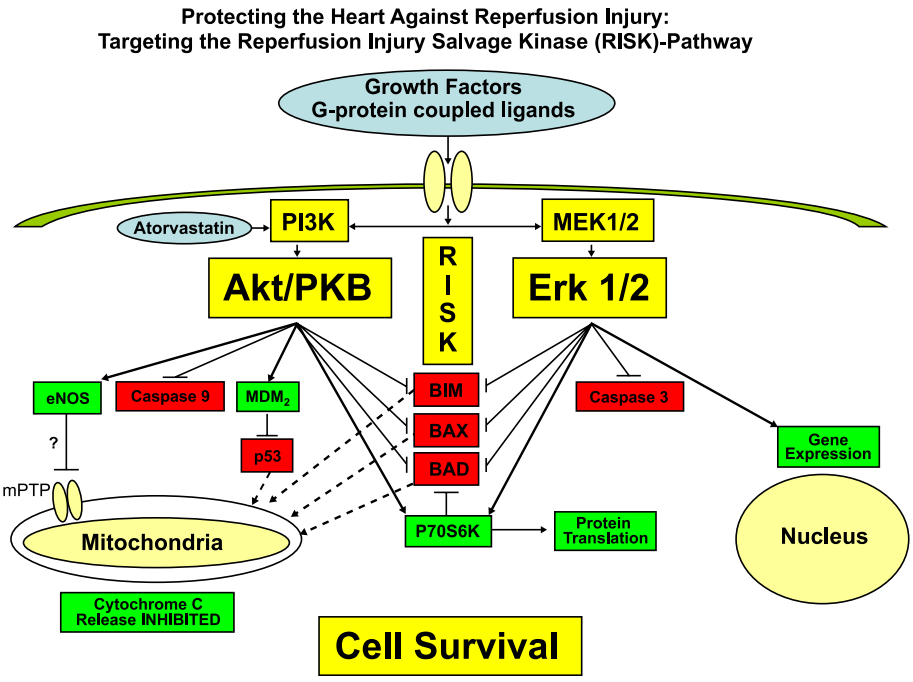

Fig. 4. Hypothetical scheme showing the potential anti-apoptotic mechanisms through which activation of the pro-survival PI3K–Akt and Erk 1/2 kinase cascades, which comprise the RISK pathway, protect the heart against lethal reperfusion-induced injury. Growth factors, G-protein-coupled receptor ligands and atorvastatin administered during the first few minutes of reperfusion initiate cardio-protection by activating the RISK pathway, which then protects against the apoptotic and necrotic components of reperfusion-induced cell death. The scheme portrays the important anti-apoptotic mechanisms that have been implicated in mediating cellular survival associated with the recruitment of these kinase cascades. Signalling through the PI3K–Akt and/or the MEK1/2–Erk 1/2 cascades results in: (1) phosphorylation and inactivation of caspases 3 and 9, which inhibits apoptosis; (2) phosphorylation and inactivation of the pro-apoptotic proteins BIM, BAX, BAD and p53, one consequence of which is to prevent the release of mitochondrial cytochrome *c* in response to an apoptotic stimulus (shown by dashed arrows); (3) phosphorylation and activation of eNOS (endothelial nitric oxide synthase), producing nitric oxide which may protect by inhibiting opening of the mitochondrial permeability transition pore (mPTP); (4) phosphorylation and activation of p70S6K which can protect by inactivating BAD or regulating protein translation; and (5) regulating the expression of genes concerned with cellular survival.

reperfusion protects the heart by recruiting the RISK pathway [100,104]. Clinical trials are required to investigate whether administration of these drugs, as an adjunct to current reperfusion therapy, offers protection against reperfusion injury following a myocardial infarction. Despite studies reporting benefit from the early administration of statins following an acute myocardial infarction [126,127], as yet no study has been undertaken which examines whether these drugs offer cardio-protection when given during the first few minutes of reperfusion following an acute myocardial infarction, as an adjunct to thrombolysis or primary angioplasty.

Recent studies have shown that fluoroscopic-guided intramyocardial injection in the pig model is a feasible and safe procedure for targeting the delivery of therapeutic agents to the area of myocardium at risk from ischaemia–reperfusion injury [128]. Therefore, the local delivery of growth factors themselves or the adenoviral vectors (carrying mutated genes which over-express growth factors) may provide a potential method for targeting and up-regulating the RISK pathway in the clinical setting of reperfusion. Alternatively for patients undergoing an anticipated episode of ischaemia–reperfusion injury, such as during CABG surgery or elective coronary angioplasty, gene transfer may be a possible method of delivering growth factors to myocardium at risk of lethal reperfusion-induced injury.

8. Conclusions

Discovering novel approaches that ameliorate reperfusion-induced myocardial injury, and can be used as an adjunct to current reperfusion strategies, may offer further salvage of viable myocardium and limit infarct size, over and above that achieved by reperfusion itself. Apoptotic cell death, which has been shown to contribute to the myocyte death sustained during ischaemia–reperfusion injury, may actually be accelerated during the reperfusion period. The pro-survival PI3K–Akt and Erk 1/2 kinase cascades are activated in response to ischaemia–reperfusion injury and initiate myocardial protection through their anti-apoptotic actions. Growth-factor-mediated up-regulation of these pro-survival kinase cascades at reperfusion has been demonstrated to protect the heart against reperfusion injury. Furthermore, other agents such as HMG-Co-A reductase inhibitors and G-protein-coupled receptor ligands such as bradykinin have also been shown to initiate cardio-protection at the time of reperfusion by activating these pro-survival kinase cas-

cades. Interestingly, activation of these pro-survival kinase cascades *prior* to ischaemia has been associated with the profound cardio-protection induced by the phenomenon of ischaemic preconditioning, suggesting perhaps that agents which activate these signalling pathways, should be able to provide protection at time of reperfusion and also precondition the myocardium. Pharmacological manipulation and activation of the anti-apoptotic pro-survival PI3K–Akt and Erk 1/2 kinase cascades, which we have termed the RISK pathway, during the early reperfusion phase, affords an opportunity to attenuate reperfusion-induced injury, thereby salvaging viable myocardium and limiting infarct size.

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