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REVIEW

Cyclic GMP and protein kinase-G in myocardial ischaemia-reperfusion: opportunities and obstacles for survival signaling

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It is clear that multiple signalling pathways regulate the critical balance between cell death and survival in myocardial ischaemia–reperfusion. Recent attention has focused on the activation of survival or salvage kinases, particularly during reperfusion, as a common mechanism of many cardioprotective interventions. The phosphatidyl inositol 3′-hydroxy kinase/Akt complex (PI3K/Akt) and p42/p44 mitogen-activated protein kinase cascades have been widely promoted in this respect but the cyclic guanosine 3′,5′-monophosphate/cGMP-dependent protein kinase (cGMP/PKG) signal transduction cassette has been less systematically investigated as a survival cascade. We propose that activation of the cGMP/PKG signalling pathway, following activation of soluble or particulate guanylate cyclases, may play a pivotal role in survival signalling in ischaemia–reperfusion, especially in the classical preconditioning, delayed preconditioning and postconditioning paradigms. The resurgence of interest in reperfusion injury, largely as a result of postconditioning-related research, has confirmed that the cGMP/PKG pathway is a pivotal salvage mechanism in reperfusion. Numerous studies suggest that the infarct-limiting effects of preconditioning and postconditioning, exogenously donated nitric oxide (NO), natriuretic peptides, phosphodiesterase inhibitors, and other diverse drugs and mediators such as HMG co-A reductase inhibitors (statins), Rho-kinase inhibitors and adrenomedullin, whether given before and during ischaemia, or specifically at the onset of reperfusion, may be mediated by activation or enhancement of the cGMP pathway, either directly or indirectly via endogenous NO generation downstream of PI3K/Akt. Putative mechanisms of protection include PKG regulation of Ca^{2+} homeostasis through the modification of sarcoplasmic reticulum Ca^{2+} uptake mechanisms, and PKG-induced opening of ATP-sensitive K^{+} channels during ischaemia and/or reperfusion. At present, significant technical obstacles in defining the precise roles played by cGMP/PKG signalling include the heavy reliance on pharmacological PKG inhibitors of uncertain selectivity, difficulties in determining PKG activity in intact tissue, and the growing recognition that intracellular compartmentalisation of the cGMP pool may contribute markedly to the nucleotide's biological actions and biochemical determination. Overall, the body of experimental evidence suggests that cGMP/PKG survival signalling ameliorates irreversible injury associated with ischaemia–reperfusion and may be a tractable therapeutic target.
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Abbreviations: ANP, atrial natriuretic peptide; BNP, B-type natriuretic peptide; cGMP, cyclic guanosine 3′,5′-monophosphate; MI, myocardial infarction; NO, nitric oxide; NOS, nitric oxide synthase; pGC, particulate guanylate cyclase; PKG, cGMP-dependent protein kinase; sGC, soluble guanylate cyclase

Introduction

Myocardial ischaemia–reperfusion injury

Ischaemic heart disease is the leading cause of human mortality with some 7.6 million deaths worldwide attributed to the disease in 2005 (Mathers and Loncar, 2006). World Health Organisation projections of global mortality as far as 2030 predict that ischaemic heart disease will retain its position as the most common cause of death worldwide and rise from the sixth place to third place as a cause of disability. Nearly all deaths from ischaemic heart disease are due to the sudden thrombotic occlusion of an atherosclerotic coronary artery. Myocardial ischaemia that develops as a result of sudden and sustained coronary occlusion is usually diagnosed as acute myocardial infarction (MI) and may result in early death by cardiac arrest due to arrhythmias or a loss of

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left ventricular contractility sufficient to impair cardiac output catastrophically. In the developed countries currently, about one-third of patients sustaining MI will die within a few hours of ischaemia onset. In patients who survive early death, sustained ischaemia will lead to irreversible cell injury within the ischaemic myocardium (the ischaemic risk zone), culminating in myocardial necrosis, which is the pathological hallmark of MI. Restoration of blood flow through the occluded artery is the mainstay of treatment for MI and the application of early reperfusion treatment to restore blood supply to the ischaemic risk zone is central to limiting the extent of irreversible injury. However, it is recognized that reperfusion may be associated with further irreversible injury (Yellon and Baxter, 2000; Downey and Cohen, 2006). Hence it is appropriate to speak of MI being an ischaemia–reperfusion pathology.

The philosophy of prompt reperfusion is grounded in the recognition that the extent of irreversible injury (i.e. infarct size) is a primary determinant of the extent of post-infarction remodeling, and the propensity for the development of chronic cardiac failure and death (Simoons *et al*., 1997). Hence, patients who survive the acute ischaemic episode but sustain a large transmural infarct are at high risk of subsequent morbidity and mortality associated with chronic cardiac failure. It follows, therefore, that any intervention that could maximize the effectiveness of reperfusion, or attenuate the rate of irreversible cell injury during either ischaemia or reperfusion, might have significant therapeutic potential. Identification of pharmacologically tractable targets to limit infarct size during ischaemia–reperfusion continues to be the focus of a considerable research effort. Here, we apply the term 'cardioprotection' specifically to interventions capable of limiting infarct size during ischaemia–reperfusion. Although we have limited our discussion to considering infarct limitation as a clinically relevant end point, pharmacological manipulation during ischaemia–reperfusion may afford protection against other end points of injury, notably arrhythmias and impairment of cardiac contractile function.

Cardioprotective signalling paradigms: preconditioning and postconditioning

The application of increasingly more refined experimental strategies has provided insights into the complex signal transduction cascades involved in regulating cardiomyocyte death and survival in ischaemia–reperfusion. Arguably, research undertaken in the context and knowledge of ischaemic preconditioning and postconditioning has fuelled the most rapid developments in this field, and has led to an appreciation of the potential for manipulation of numerous autacoid mediators, intracellular signalling pathways and functional target proteins.

Preconditioning the myocardium with brief periods of ischaemia, applied before the onset of prolonged ischaemia (termed 'index ischaemia' in experimental studies), delays the development of irreversible injury and ultimately reduces infarct size (Murry *et al*., 1986; Yellon *et al*., 1998; Yellon and Downey, 2003; Heusch, 2004). More recently, a growing appreciation of the protective potential of postconditioning, a method that modifies the conditions of early reperfusion through the introduction of very brief episodes of ischaemia at the onset of reperfusion, has re-ignited interest in early reperfusion as a potential target for tissue salvage. Indeed, recent developments suggest that reperfusion injury may contribute to final infarct size to a far greater extent than was previously supposed. A significant development has been the recognition that preconditioning and postconditioning share similar mechanisms and that protection may be predominantly conferred during the reperfusion phase (Hausenloy and Yellon, 2004).

Although precise mechanisms are far from clear, it is now known that multiple signalling pathways regulate the critical balance between cell death and cell survival in ischaemia–reperfusion. Kinase signalling pathways that have been the subject of extensive investigation and comment with regard to cardioprotection include protein kinase C (PKC) isoforms, p38 mitogen-activated protein kinase (p38 MAPK) isoforms, cyclic adenosine mono phosphate (cAMP)-dependent protein kinase, the phosphatidyl inositol 3′-hydroxy kinase/Akt complex (PI3K/Akt) and the p42/p44 MAPKs (for a comprehensive review, see Hausenloy and Yellon, 2004). Although pathways associated with cyclic guanosine 3′,5′-monophosphate (cGMP) generation, such as the nitric oxide (NO)-soluble guanylyl cyclase (sGC) cascade, have been extensively studied (Bolli, 2001; Cohen *et al*., 2006), the role of cGMP-dependent protein kinase (PKG) as a signalling intermediate in this pathway has received less systematic analysis and critical attention until relatively recently (Abdallah *et al*., 2005; Burley and Baxter, 2005; Cuong *et al*., 2006; Das *et al*., 2006). The focus of this review is the cGMP pathway as a survival cascade in ischaemia–reperfusion.

Cyclic guanosine 3′,5′-monophosphate

cGMP generation

cGMP is a second messenger signalling molecule first described in 1963 (Ashman *et al*., 1963). cGMP is generated from the cytosolic purine nucleotide guanosine triphosphate (GTP) by two distinct enzymes: the cytoplasmic heterodimeric haemoprotein sGC activated by NO and carbon monoxide (CO); and the transmembrane receptor particulate guanylate cyclases (pGCs, GC or natriuretic peptide receptor (NPR)) that act as functional receptors for the natriuretic peptides (Lucas *et al*., 2000; Hussain *et al*., 2001; Feil *et al*., 2003; Kuhn, 2004).

NO and CO activate sGC via different mechanisms: the former directly binds the ferrous core of the enzyme, which leads to creation of a protoporphyrin IX-like structure (a potent activator of sGC), whereas CO yields a hexacoordinated complex consisting of iron, imidazole and CO axial ligands (Lucas *et al*., 2000). Oligomerization and phosphorylation enables the extracellular binding domains of pGC to remain in a high-affinity state, thus priming the cytoplasmic domain to respond to ligand receptor interaction. Binding of natriuretic peptide to the single high-affinity extracellular binding domain facilitates ATP linkage to the kinase

homology domain of pGC (Lucas *et al*., 2000). GTP binds to a single catalytic site on sGC and two catalytic sites on pGC. The α- and β-subunits of the guanylate cyclases cause cleavage of the α-phosphoanhydride bond of GTP yielding cGMP and pyrophosphate (Lucas *et al*., 2000).

Subcellular localization of cGMP

There is emerging evidence that cGMP distribution within cells is not uniform and that intracellular cGMP may be functionally compartmentalized, accounting for differential effects of cGMP within the cell depending on whether it is generated from sGC or pGC (Zolle *et al*., 2000; Hart *et al*., 2001; Su *et al*., 2005). For example, B-type natriuretic peptide (BNP) has been shown to potentiate ATP- and thapsigargin-stimulated rise in cytosolic-free Ca^{2+} in human epithelial cells, and causes partial inhibition of cation influx (Zolle *et al*., 2000). In contrast the NO donor, sodium nitroprusside, caused an increase in re-uptake of Ca^{2+} into the sarcoplasmic reticulum (SR) without affecting cation influx or Ca^{2+} efflux, thus suggesting that localized pools of cGMP play different roles in regulating Ca^{2+} homeostasis in cells (Zolle *et al*., 2000). Hart *et al*. (2001) showed that NO and BNP modulated left-ventricular function in dogs with congestive chronic heart failure by preserving diastolic function through elevation of cGMP. In contrast, β-adrenergic responsiveness to dobutamine was dampened by NO but not BNP, illustrating the differential effects of sGC and pGC-derived cGMP on cardiac inotropy. Although C-type natriuretic peptide (CNP) generated negligible amounts of cGMP compared to S-nitroso-*N*-acetyl-penicillamine (SNAP), Su *et al*. (2005) found that CNP reduced intracellular Ca^{2+} transients in ventricular myocytes, whereas SNAP had little or no effect. Using cultured vascular smooth muscle cells, Piggott *et al*. (2006) demonstrated that Atrial natriuretic peptide (ANP) activated cyclic nucleotide-gated ion channels (CNG) more readily than SNAP, although SNAP generated higher levels of cGMP compared to ANP in the same preparation. These examples constitute robust evidence for differential responses to sGC and pGC stimulation, and may be one of the underlying reasons for inconsistent results provided by investigators on the effectiveness of NO donors and precursors in reducing infarct size and cell death, in *in vivo*, *ex vivo* and *in vitro* models of myocardial ischaemia–reperfusion injury discussed in detail below.

cGMP actions

At least three classes of proteins bind cGMP and facilitate its signal transduction roles: cGMP-dependent protein kinases or protein kinase-G (PKG), the cGMP-regulated phosphodiesterases (PDEs) and the CNG (Lucas *et al*., 2000; Feil *et al*., 2003; Su *et al*., 2005).

In the mammalian cardiovascular system, the biological actions of elevated intracellular concentration of cGMP are numerous and diverse. They include vascular smooth muscle relaxation; regulation of ion transport, contributing to electrolyte/ion homeostasis and vascular cell permeability; inhibition of platelet activation mechanisms; regulation of cell growth, differentiation and apoptosis; and cardiac myocyte contractility (inotropy) (Kojda and Kottenberg, 1999; Lucas *et al*., 2000; Feil and Kemp-Harper, 2006). These effects in the cardiovascular system are in part attributable to the principal intracellular mediator of cGMP, PKG, and in part due to alterations in cAMP and cGMP PDEs and CNG channel activity (Kuo and Greengard, 1970; Lohmann *et al*., 1997; Kojda and Kottenberg, 1999; Lucas *et al*., 2000; Lincoln *et al*., 2001; Wall *et al*., 2003).

cGMP changes in myocardial ischaemia–reperfusion

Studies have shown cGMP levels to increase during ischaemia (Depre and Hue, 1994; Szilvassy *et al*., 1994; Lochner *et al*., 1998; Penna *et al*., 2006). In the isolated working rat heart, 10 min no-flow ischaemia and anoxia caused a marked increase ($\approx$30–50%) in myocardial cGMP content (Depre and Hue, 1994). Szilvassy *et al*. (1994) provided the first biochemical evidence that preconditioning-induced protection against ischaemia–reperfusion injury (using recovery of cardiac contractile function as the experimental end point) was associated with elevated myocardial cGMP content. Lochner *et al*. (1998) also characterized the changes in myocardial cGMP during ischaemia–reperfusion. They found that preconditioning with 5-min period of global ischaemia increased myocardial cGMP content, which eventually normalized upon reperfusion. They also found cGMP content to be greater in preconditioned hearts compared to non-preconditioned hearts (Lochner *et al*., 1998). cGMP release is also augmented in the isolated rat heart subjected to postconditioning (Penna *et al*., 2006). These studies support the emerging paradigm that during ischaemia, elevation of cGMP occurs and that this increase in cGMP content is an endogenous cardioprotective response during ischaemia–reperfusion, since it is augmented further by preconditioning and postconditioning. During ischaemia, the ability of pGC to synthesise cGMP is reduced due to low intracellular pH. Hence, cardiomyocyte cGMP content during ischaemia is largely dependent on NO generation (Agullo *et al*., 2003). However, the relationships between myocardial NO and cGMP elevation are likely to exhibit a degree of complexity, as seen in smooth muscle cells (Csont *et al*., 1998, 2003; Csont and Ferdinandy, 2005). Furthermore, recent work suggests that sGC expression and activity may be attenuated by reactive oxygen species in rat aortic smooth muscle cells (Gerassimou *et al*., 2007). The extent to which this phenomenon contributes to sGC activity in myocardium is unknown. However, the reduction of reactive oxygen species (ROS) generation during index ischaemia–reperfusion in preconditioned hearts could conceivably lead to enhancement of sGC activity and cGMP content after preconditoning.

cGMP-dependent protein kinase

PKG is a cytosolic 75-kDa homodimer first located and identified in lobster tail muscle by Kuo and co-workers (Kuo *et al*., 1970; Kuo and Greengard, 1970). Two genes have been found in mammalian cells coding for two PKG homologues: type I (PKG-Iα and -Iβ) and type II (PKG-II) (Lohmann *et al*.,

1997; Lucas *et al*., 2000; Feil *et al*., 2003; Wall *et al*., 2003). The two closely related isoforms of PKG-I are not co-expressed in the same tissue and it is the PKG-Iα isoform that is responsible for transducing the effects of sGC- and pGC-derived cGMP in cardiovascular homeostasis (Feil and Kemp-Harper, 2006). PKG-Iα has been detected in cardiomyocytes, vasculature, lung, kidney, adrenal glands and cerebellum, whereas PKG-Iβ isoform expression has been found in the uterus (Wall *et al*., 2003; D'Souza *et al*., 2004). PKG consists of three functional domains. The N-terminal domain mediates dimerization and suppression of kinase activity in the absence of cGMP. The binding domain contains the high-affinity cGMP-binding site A, and the low-affinity cGMP-binding site B. The Mg^{2+}/ATP and target protein binding domain cause the catalytic transfer of phosphate from ATP to a serine or threonine residue on target proteins (Lohmann *et al*., 1997; Lucas *et al*., 2000; Feil *et al*., 2003; Wall *et al*., 2003).

Regulation of PKG activity

Biological responses to the cGMP/PKG signalling cascade are regulated by the cyclic nucleotide PDEs, especially under conditions of low Ca^{2+} (Friebe and Koesling, 2003; Rybalkin *et al*., 2003). The PDEs are not necessarily colocalized with each other, and can therefore serve different functional compartments in cells, determining subtle subcellular organization of cGMP and other cyclic nucleotides (Lucas *et al*., 2000; Rybalkin *et al*., 2003; Burnett, 2005). Although cGMP is hydrolysed by the PDE families 1, 2, 3, 5, 6 and 10, it is predominantly cGMP binding cGMP-specific phosphodiesterase (PDE5), which is responsible for modulating the intracellular concentrations of cGMP produced by NO and the natriuretic peptides in cardiovascular tissues, thus ultimately affecting cellular and functional responses in the myocardium (Lucas *et al*., 2000; Burnett, 2005). Increasing cGMP concentration leads to activation of PDE5 via interaction between cGMP and the PDE GAF catalytic domain. This in turn causes the cleavage of a phosphodiester bond, hydrolysing cGMP to its corresponding inactive nucleotide 5′-GMP (Lucas *et al*., 2000). Phosphorylation of PDE5 by PKG serves to increase its cGMP affinity, and represents an alternative mode of regulatory feedback inhibition within the cGMP/PKG signalling cascade, thus normalizing levels of cGMP (Lucas *et al*., 2000; Friebe and Koesling, 2003; Rybalkin *et al*., 2003).

PKG-I substrates in the cardiovascular system

There are numerous biological substrates for PKG-I in the cardiovascular system. Among the most important of these are:

(i) phospholamban, inositol-1,4,5-trisphosphate (IP_3) receptor and the ryanodine receptor (RyR) on the SR, regulating Ca^{2+} homeostasis (Takasago *et al*., 1991; Lucas *et al*., 2000; Lincoln *et al*., 2001);
(ii) the vasodilator-stimulated phosphoprotein regulating thin filament involved in smooth muscle cell contraction, platelet and neutrophil activation (Lucas *et al*., 2000);
(iii) troponin, regulating cardiac muscle contraction (Satoh and Makino, 2001; Layland *et al*., 2002);
(iv) the thromboxane A_2 receptor in platelets regulating platelet activation (Lucas *et al*., 2000);
(v) the L-type Ca^{2+} channel and Ca^{2+}-activated potassium (BK_{ca}) channel, regulating smooth muscle tone and heart contractility, and maybe cell death (apoptosis) (Fukao *et al*., 1999; Lucas *et al*., 2000; Nara *et al*., 2000; Lincoln *et al*., 2001; Xu *et al*., 2002);
(vi) the myosin light chain phosphatase, inducing vascular smooth muscle relaxation and vasodilatation (Piper *et al*., 1998, 2004; Piper and Garcia-Dorado, 1999; Xu *et al*., 2002; Zhang *et al*., 2005);
(vii) Another indirect target for PKG-I relevant to ischaemia-reperfusion is the ATP-sensitive K^+ channel (K_{ATP}), downstream of mitochondrial PKC-ε isoforms (Costa *et al*., 2005, 2006).

Cardioprotective effects of NO/sGC pathway

During the last 15 years there have been many studies investigating the roles played by NO in mediating and attenuating myocardial ischaemia–reperfusion injury (Bolli, 2001; Dawn and Bolli, 2002; Jones and Bolli, 2006). Although there is little debate that NO is cardioprotective, this has proved to be a complex area of research, since NO has the potential to be both beneficial in attenuating ischaemia–reperfusion and deleterious due its propensity to form reactive nitrogen species. Moreover, the complexities surrounding the role of NO in ischaemia–reperfusion are further increased when one considers the diversity of experimental models (species, end points of protection, early versus delayed protection, effects during ischaemia versus effects at reperfusion) and the numerous potential pathways of injury and protection. It is important to note here that NO may exert biological actions through mechanisms other than activation of sGC and elevation of cGMP (Wanstall *et al*., 2005). For example, direct nitrosylation of proteins by NO may significantly modify their function (Choi *et al*., 2002).

Bolli (2001) examined 92 studies conducted between 1990 and 2001 focusing on the role of NO in myocardial ischaemia, and reported that three quarters of these provided evidence that NO (either endogenously generated or exogenously derived from NO donors and precursors such as L-arginine) was cardioprotective. Many of these studies found that inhibition or genetic manipulation of endogenous nitric oxide synthase (NOS) activity exacerbated myocardial ischaemia–reperfusion injury, whereas NO significantly reduced this injury (Bolli, 2001). However, there is a model-dependency in NO's actions. For example, pharmacological inhibition of either NOS or sGC exacerbates ischaemia–reperfusion arrhythmias, whereas administration of NO donors tends to diminish the severity of these arrhythmias (Vegh *et al*., 1993; Pabla and Curtis, 1996). However, in the majority of studies using infarct size as the end point of ischaemia–reperfusion injury, pharmacological inhibition of basal NOS or sGC activities or genetic deletion of NOS expression does not exacerbate infarct development

per se, although administration of NO donors before ischaemia reliably reduces infarct size; hence, producing a preconditioning-like state (Bolli, 2001; Schulz *et al*., 2004; Cohen *et al*., 2006; Jones and Bolli, 2006). Mouse hearts overexpressing endothelial nitric oxide synthase (eNOS) were demonstrated to confer a maximal protective state comparable to that exhibited by genetically normal mouse hearts subjected to preconditioning suggesting that these hearts are maximally protected against infarction by their elevated endogenous NO levels (du Toit *et al*., 2007).

NO in classical and delayed preconditioning

The role of endogenous NO in mediating classical preconditioning has been extensively reviewed elsewhere (Ferdinandy and Schulz, 2003; Yellon and Downey, 2003; Downey and Cohen, 2006). Here, we summarize the major strands of evidence. Experimental studies using NO donors such as SNAP and diethylenetriamine-nitric oxide (DETA-NO), or the NO precursor/NOS substrate L-arginine given before ischaemia, have generally found these compounds to limit infarct size and improve post-ischaemic functional recovery, mimicking classical preconditioning (Horimoto *et al*., 2000; Nakano *et al*., 2000; Suematsu *et al*., 2001; Gourine *et al*., 2002; Lellouche *et al*., 2002; Lochner *et al*., 2002; Bell *et al*., 2003; Otani *et al*., 2003; Pagliaro *et al*., 2003; Qin *et al*., 2004; Xu *et al*., 2004).

A number of studies showed that inhibition of NOS during preconditioning abolishes the protective effects of classical preconditioning, especially protection against the development of malignant arrhythmias (Vegh *et al*., 1993) and post-ischaemic contractile dysfunction (Lochner *et al*., 2002). Ferdinandy and Schulz (2003) postulated that in these models where classical preconditioning is dependent on NO synthesis, NO and superoxide (O_2^-) combine to generate peroxynitrite ($ONOO^-$), which is a trigger of an intracellular signal transduction cascade that leads to attenuation of NO, O_2^- and $ONOO^-$ generation in the preconditioned heart during a subsequent injurious ischaemia–reperfusion episode. The role of endogenous NO in mediating the cardioprotective effects of classical preconditioning against infarction was historically an area of controversy and confusion, which is only now being resolved. For example, Nakano *et al*. (2000) reported that N^G-nitro-L-arginine methyl ester (L-NAME) did not attenuate the infarct limiting effects of classical preconditioning in rabbit isolated heart. Furthermore, infarct limitation induced by pharmacological preconditioning with bradykinin was found to be both NO-independent (Goto *et al*., 1995) and NO-dependent (Oldenburg *et al*., 2004) in the same rabbit isolated heart preparation. The gathering of further data strongly supports a role of NO downstream of Akt phosphorylation of eNOS in the mechanism of classical preconditioning against infarction (Cohen *et al*., 2006).

Induction of delayed protection by NO donors and the role of endogenous NO in triggering and mediating delayed preconditioning is an increasingly recognized aspect of NO biology. Bolli *et al*. (1997) have provided extensive evidence that endogenous NO is a trigger of delayed preconditioning. Evidence corroborating the NO hypothesis of delayed preconditioning is reviewed elsewhere (Jones and Bolli, 2006). Briefly, the generation of NO during preconditioning plays an essential role in triggering a multiple-kinase signal transduction cascade that results in transcriptional upregulation of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) many hours after the preconditioning stimulus has been applied. Both these proteins are obligatory mediators of the delayed cardioprotection against infarction and reperfusion-induced contractile dysfunction (stunning). Thus, NO either as a result of preconditioning or administration of donor compounds induces late protection through upregulation of iNOS (Guo *et al*., 2005). Interestingly, Kodani *et al*. (2002) showed that myocardial cGMP content was markedly elevated in conscious rabbits 24 h after preconditioning. Administration of 1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), a selective inhibitor of sGC, at the time of preconditioning did not attenuate the protection afforded by preconditioning 24 h later. Thus, NO acting during the trigger phase of delayed preconditioning is cGMP-independent. However, ODQ given before the index ischaemia–reperfusion insult abolished the protective effects of delayed preconditioning, suggesting that NO formed from iNOS acts in a cGMP-dependent manner to mediate protection. However, other studies examining the role of NO in iNOS-dependent delayed protection suggest that the mechanism involved may be cGMP-independent. Atar *et al*. (2006) proposed that iNOS induced s-nitrosylation of COX-2, to mediate atorvastatin-induced delayed preconditioning.

Endogenous NO and protection against reperfusion injury

Postconditioning was introduced by Zhao *et al*. (2003) as a new cardioprotective phenomenon. It is debatable whether the application of fleeting periods of coronary artery occlusion applied at the onset of reperfusion is genuinely a new form of protection or represents a rediscovery of the benefits of modified, staged or controlled reperfusion (Okamoto *et al*., 1986; Vinten-Johansen *et al*., 1986; Allen *et al*., 1993; Sato *et al*., 1997; Heusch, 2004). However, postconditioning has reawakened interest in reperfusion injury and the molecular mechanisms of cell injury and survival that are activated during reperfusion.

Yang *et al*. (2004, 2005) have provided evidence to suggest that cardioprotection provided by postconditioning is abolished by L-NAME and ODQ, thus implicating NOS isoforms and sGC as important elements of the signal transduction cascade of postconditioning. Another study found that postconditioning causes a marked increase in eNOS phosphorylation, further suggesting that increased NO production is important in the protection provided by postconditioning (Tsang *et al*., 2004). In addition, it was shown that this phosphorylation was Akt-dependent, since eNOS phosphorylation was attenuated in hearts subjected to postconditioning and concomitantly treated with the PI3K/Akt inhibitors wortmannin and LY294002 (Hausenloy *et al*., 2004, 2005; Hausenloy and Yellon, 2004; Tsang *et al*., 2004, 2005; Cohen *et al*., 2006).

NO generated downstream of PI3K/Akt appears to be a key signalling element of the reperfusion salvage pathway activated by many classes of agents capable of protecting

the myocardium at reperfusion. In some cases (cf. adrenomedullin and insulin below), the dependency of the protection on sGC/cGMP/PKG downstream of NO has been established. Several peptide hormones, other than the natriuretic peptides, have been shown to exert marked cardioprotection when given at reperfusion. These include adrenomedullin, bradykinin and insulin. The mechanisms of protection are known to involve PI3K/Akt and NOS/NO signal transduction (Hausenloy and Yellon, 2006). The infarct sparing effect of exogenous adrenomedullin administered during early reperfusion in the rat isolated heart was associated with augmented Akt phosphorylation. Furthermore, the infarct-limiting effect of adrenomedullin given at reperfusion was blunted by L-NAME in rat heart (Hamid and Baxter, 2005). In addition, in mouse heart the protection was associated with increased NO_2^- production and cGMP accumulation, and was abrogated by ODQ (Hamid *et al*., 2007b), indicating the NO- and cGMP-dependency of adrenomedullin's protective action. Bradykinin administration at reperfusion limited infarct size and was associated with phosphorylation of Akt and eNOS, in a mouse isolated heart model of reperfusion injury (Bell and Yellon, 2003b). Abdallah *et al*. (2006) demonstrated the underlying mechanism of insulin-induced protection of reoxygenated rat ventricular myocytes, involves activation of a survival pathway comprising PI3K, eNOS and PKG.

Hydroxymethylglutarate coenzyme A reductase inhibitors (statins) have been shown to have beneficial pleiotropic effects, independent of cholesterol lowering (Liao and Laufs, 2005). Some evidence suggests that cardioprotection induced by statins against myocardial reperfusion injury is mediated by survival kinases (PI3K/Akt and p44/p42 MAPK) and endogenous NO (Bell and Yellon, 2003a; Efthymiou *et al*., 2005). Atorvastatin-induced protection against myocardial reperfusion injury characterized by a significant reduction in infarct size was found to be wortmannin sensitive. Furthermore, the infarct limiting effect of atorvastatin was lost in $eNOS^{-/-}$ mice (Bell and Yellon, 2003a; Efthymiou *et al*., 2005). Simvastatin given intravenously just before reperfusion in anaesthetised rats induced a marked reduction in infarct size, increased myocardial PI3K, Akt and eNOS phosphorylation (Wolfrum *et al*., 2004). In addition, the cardioprotective effect of simvastatin was blunted by L-NAME, suggesting that endogenous NO production is important in simvastatin-induced protection against myocardial reperfusion injury (Wolfrum *et al*., 2004).

Rho-associated coiled-coil protein kinase-1 (Rho kinase) may act as a negative regulator of PI3K/Akt, possibly via activation of the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) (Mocanu and Yellon, 2007). The beneficial effect of Rho kinase inhibitors on infarct size, which is independent of coronary vasodilatation, has been demonstrated in experimental models of MI (Hamid *et al*., 2007a). Both fasudil and Y27632, administered at reperfusion, limited infarct size in the rat isolated heart subjected to ischaemia–reperfusion. This protection was reversed by wortmannin, and L-NAME, confirming a protective mechanism involving PI3K/Akt and NOS/NO (Hamid *et al*., 2007a). Whether the beneficial action of NO in this case is subsequently mediated via cGMP/PKG is unknown but this would seem likely.

Exogenous NO and reperfusion injury

Although pre-ischaemic administration of synthetic NO donors has been shown to be beneficial in limiting myocardial injury, the benefits of NO donors in providing acute salvage at reperfusion are less clear and warrant further investigation. The cysteine containing NO donor SP/W-5186, given just before reperfusion, exerted marked cardiac protective effects in rabbit intact hearts, shown as improvement in cardiac function, decreased plasma creatine kinase and reduced infarct size (Liu *et al*., 1998). Comparably, Ma *et al*. (1999) found that the *S*-nitrosoglutathione, but not Angell's salt (a source of NO_2^-) given just before reperfusion, markedly attenuated infarct size, in rabbit intact hearts. Bell and Yellon (2003b) reported that bradykinin administered at early reperfusion induced infarct limitation in mouse heart in an eNOS/PI3K/Akt-dependent manner. This protection was recapitulated in eNOS knockout mouse heart by administration of SNAP 1 μM. In contradiction to this study, Burley and Baxter (2007) found that SNAP (1, 2, 5 and 10 μM) given during early reperfusion, provided negligible protection of the rat isolated heart. The reasons why NO donors are not consistently protective when administered at reperfusion even though 8-bromo-cGMP (Burley and Baxter, 2005) and BNP (Burley and Baxter, 2007) are protective under similar treatment conditions are not known. It is possible that ROS generated during early reperfusion scavenge the NO generated by some NO donors, thus impairing their cytoprotective action (Taimor *et al*., 2000).

Cardioprotective effect of the natriuretic peptide/pGC pathway

In the classical physiological and clinical descriptions, the cardiac release of ANP and BNP increases in response to chamber volume overload and myocardial stretch (D'Souza *et al*., 2004). However, there is now good evidence from both clinical and experimental studies that the natriuretic peptides are released rapidly in response to myocardial ischaemia. ANP and BNP levels are raised in patients in the early phase of acute MI, and even in response to brief coronary occlusion during percutaneous coronary intervention (Burley *et al*., 2007). Release of BNP in response to myocardial ischaemia has been reported in experimental models where the magnitude of release following global ischaemia correlates with the duration of ischaemia (D'Souza *et al*., 2004; Nishikimi *et al*., 2006). This pattern of cardiac release of natriuretic peptides in response to ischaemia is common to several families of cardiac peptide hormones. Although these peptides exert pleiotropic actions, regulating many responses to impaired cardiac output through endocrine actions in the circulation, we have proposed that their early release in myocardial ischaemia may be indicative of their roles as cardioprotective autacoids (Burley *et al*., 2007).

Pre-ischaemic treatment with natriuretic peptides

The natriuretic peptides limit infarction in *in vivo* and *ex vivo* models of myocardial ischaemia–reperfusion injury. Using a rat isolated heart model of infarction, D'Souza *et al*. (2003)

found that BNP given before and during an episode of coronary artery occlusion markedly reduced infarction in a concentration-dependent manner. This protective effect was abrogated by the K_{ATP} blockers, glibenclamide and 5-hydroxydecanoic acid (5-HD), suggesting that K_{ATP} channel opening is important in the protection provided by BNP. The protective action of BNP has been corroborated by Giricz *et al*. (2006) using an isolated cardiomyocyte preparation where it was demonstrated that marked protection against hypoxia–reoxygenation injury in rat ventricular myocytes was conferred by treatment with BNP. Curiously, the infarct sparing effect of BNP in rat heart was abolished by co-perfusion with L-NAME and ODQ, suggesting that protection afforded by BNP has an NO/sGC component (D'Souza *et al*., 2004). The NO/sGC dependency of the protective action of BNP has been confirmed in another study (Ren *et al*., 2007) and has also been observed with ANP. Okawa *et al*. (2003) demonstrated that ANP is effective in limiting infarct size in the isolated rat heart and this protective effect was reversed by L-NAME, 5-HD and the PKC inhibitor chelerythrine (Okawa *et al*., 2003). Thus, the protective action of natriuretic peptides in ischaemia–reperfusion is not simply due to the elevated concentration of pGC-derived cGMP.

Cardioprotective effects of natriuretic peptides at reperfusion

ANP and the related peptide, urodilatin, given at reperfusion, were shown to normalize intracellular cGMP and attenuate necrosis in an *in vivo* porcine model of infarction (Padilla *et al*., 2001). Inserte *et al*. (2000) showed that urodilatin limited reperfusion injury in the rat isolated heart preparation. In rabbit isolated heart, Yang *et al*. (2006) showed that ANP given at reperfusion limited infarct size, via mechanisms involving activation of PI3K/Akt and extracellular signal-regulated kinase 1/2. It is very conceivable that these effects are mediated by the high-affinity binding of natriuretic peptides (NPs) to natriuretic peptide receptor-C (NPR-C) that is traditionally viewed as a clearance receptor. However, it is believed that this receptor is Gi-protein coupled, and may be responsible for some of the biological actions of natriuretic peptides (Hobbs *et al*., 2004; Anand-Srivastava, 2005; Scotland *et al*., 2005) by possibly recruiting signalling pathway(s) including PI3K (see Figure 1) (Anand-Srivastava, 2005; Huang *et al*., 2006). BNP exerts an identical protective action when administered at reperfusion in the rat isolated heart (Burley and Baxter, 2007). In isolated cardiac myocytes subjected to hypoxia–reoxygenation, ANP, urodilatin and 8-Bromo-cGMP protected against reoxygenation induced hypercontracture (putative reperfusion injury) (Hempel *et al*., 1997). The protective action of natriuretic peptides in cells was confirmed by Abdallah *et al*. (2005), who used a cardiomyocyte model of reoxygenation injury to demonstrate that selective PKG activators and urodilatin cause an increase in sarcoplasmic reticulum ATPase (SERCA) activity, thus inhibiting Ca^{2+}-induced hypercontracture. ANP has also been demonstrated to exert powerful reperfusion injury limiting effects when given as an adjunct to percutaneous coronary intervention (coronary angioplasty) for ST-segment elevation MI (Kitakaze *et al*., 2006). In the prospective, randomized, placebo-controlled Japan-Working Groups of Acute Myocardial Infarction for the Reduction of Necrotic Damage (J-WIND) study, MI patients undergoing reperfusion therapy received human ANP (carperitide; $0.025\,\mu g\,kg^{-1}\,min^{-1}$ for 3 days; $n=290$) or vehicle (5% glucose solution

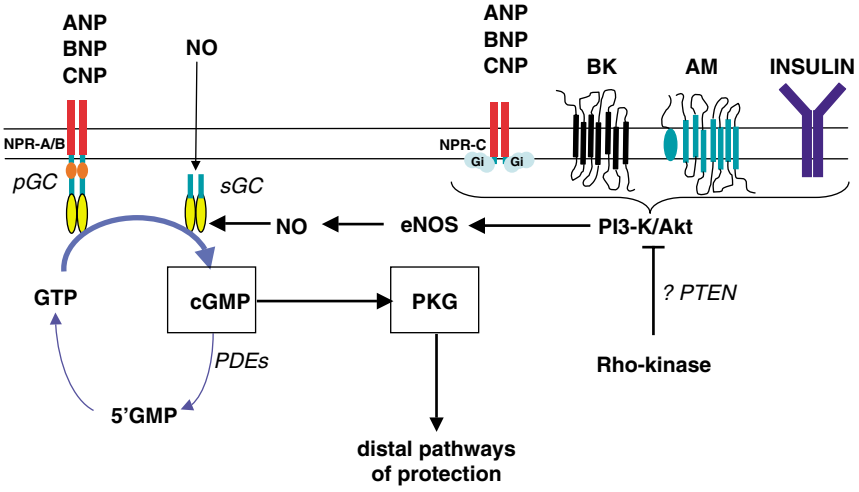

Figure 1 Direct and indirect pathways leading to cGMP-dependent protein kinase (PKG) activation by cardioprotective autacoids. Activation of particulate and soluble guanylate cyclases (pGC and sGC) by natriuretic peptides and nitric oxide (NO) catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine 3′,5′-monophosphate (cGMP). PKG is a major effector of cGMP actions; kinase activity is promoted when cGMP binds to high- and low-affinity binding sites. cGMP concentration is regulated within intracellular compartments by discrete spatial localization and activity of cGMP-dependent phosphodiesterases (PDEs). NO can act in an autocrine/paracrine manner after diffusion through the extracellular space. It may also be generated within the cardiac myocytes downstream of phosphatidyl inositol 3′-hydroxy kinase/Akt complex (PI3K/Akt) activation in response to natriuretic peptides (NPs) (binding to natriuretic peptide receptor-C (NPR-C)) and a number of autacoid ligands, including the peptides bradykinin (BK), adrenomedullin (AM) and insulin. Increasing experimental evidence supports the involvement of cGMP/PKG signalling, downstream of the PI3K/Akt/NO survival cassette in mediating the cardioprotective actions of these peptides (for details see text). Rho-associated coiled-coil protein kinase-1 (Rho-kinase) negatively regulates PI3K/Akt, possibly by promoting the activity of the phosphatase and tensin homologue deleted on chromosome 10 (PTEN).

for 3 days; $n = 313$). ANP was demonstrated to reduce plasma creatine kinase compared with placebo by 14.7%, increase left ventricular ejection fraction by 5.1% and to reduce 'reperfusion injury' (defined as malignant arrhythmias, re-elevation of ST-segment or worsening chest pain) by 25.9% (Kitakaze *et al*., 2006).

Cardioprotective effects of phosphodiesterase inhibitors

As described above, the major pathway for cGMP degradation in myocardium is PDE5. The effects of augmenting cGMP accumulation, through application of PDE5 inhibitors during ischaemia–reperfusion, has been relatively little explored. Early evidence obtained with selective inhibitors such as zaprinast (Pabla *et al*., 1995) or the mixed PDE1/PDE5 inhibitor cicletanine (Szilvassy *et al*., 1993) suggested that PDE inhibition attenuated reperfusion-induced ventricular fibrillation and contractile failure. More recent studies have shown that the selective PDE5 inhibitor sildenafil reduced infarct size *in vivo* (Ockaili *et al*., 2002; Bremer *et al*., 2005; Rosanio *et al*., 2006) and *ex vivo* (Salloum *et al*., 2003) when given before ischaemia. Transient administration of sildenafil also induces an adaptive phenomenon akin to delayed preconditioning that is dependent on induction of iNOS (Kukreja, 2007). Although the mechanism is unclear, it is proposed that transient elevation of cGMP may induce iNOS gene expression, possibly via activation of PKG-I (Das *et al*., 2006). Salloum *et al*. (2007) demonstrated the infarct limiting effect of both sildenafil and vardenafil, when administered at reperfusion following ischaemia in rabbit intact hearts. Furthermore, the protective effects of both PDE inhibitors were abolished by the mitochondrial K_{ATP} (mK_{ATP}) channel inhibitor 5-HD, suggesting that both sildenafil and vardenafil protect the ischaemic myocardium against reperfusion injury via opening of mK_{ATP} channels (Salloum *et al*., 2007). A study by Takimoto *et al*. (2005) showed that chronic inhibition of PDE5 with sildenafil reduced myocardial hypertrophy remodelling and maintained heart function in intact mouse hearts subjected to transverse aortic constriction and sustained pressure overload. Interestingly, these workers found that sildenafil did not produce a significant increase in cGMP levels compared to baseline, but conversely caused a marked increase in PKG-I activity (Takimoto *et al*., 2005). This may be indicative of special compartmentalized cGMP signalling involved in suppressing myocardial hypertrophy (Mendelsohn, 2005; Takimoto *et al*., 2005).

Pro-survival mechanisms of the cGMP/PKG pathway

Regulation of Ca^{2+} homeostasis at reperfusion

Mechanical stresses (e.g. ischaemic rigor contracture) and chemical stresses (e.g. ROS) generated during ischaemia–reperfusion cause sarcolemmal and cytoskeletal fragility and render cardiomyocytes more susceptible to irreversible injury and cell death during early reperfusion (Inserte *et al*., 2002; Piper *et al*., 2004). Replenished ATP levels (re-energisation) and increased cytosolic Ca^{2+}, rapid normalization of cell pH and cell osmolality, are considered to be the causes of lethal reperfusion injury to cardiomyocytes (Piper and Garcia-Dorado, 1999; Rodrigo and Standen, 2005). Cardiomyocyte hypercontracture, caused by ischaemia-induced Ca^{2+} overload and rapid recovery of oxidative phosphorylation, is proposed to be a major trigger of cardiomyocyte death during the early phase of reperfusion (Vittone *et al*., 2002; Piper *et al*., 2004). Piper *et al*. (2004) have proposed a mechanism of cGMP/PKG-mediated protection against cardiomyocyte hypercontracture, involving cGMP/PKG-induced re-uptake of Ca^{2+} into the SR via interaction with SERCA, and desensitization of myofibrils to Ca^{2+}. In the myocardium, SERCA-2 is regulated by phospholamban, which is phosphorylated by PKG at Ser^{16} (Vittone *et al*., 2002; Zhang *et al*., 2005). Several putative targets have been proposed for PKG for the control of Ca^{2+} homeostasis in cardiomyocytes. These include the sarcolemmal L-type Ca^{2+} channel (dihydropyridine receptor), which upon phosphorylation by PKG, inhibits Ca^{2+} entry during phase 2 of the action potential; the Ca^{2+}-activated potassium channel (BK channel), which upon sarcolemmal hyperpolarisation, decreases influx of Ca^{2+} through the L-type Ca^{2+} channel; phospholamban and the RyR, which allows the SR to sequester Ca^{2+}; the IP_3 receptor and the IP_3 receptor-associated PKG-I substrate, which reduces Ca^{2+} release from the SR (Kwan *et al*., 2000; Lucas *et al*., 2000; Lincoln *et al*., 2001; Zucchi *et al*., 2001; Piper *et al*., 2004).

cGMP may be a regulator of intracellular Ca^{2+}-independent of PKG activation, via interaction with CNGs (Lucas *et al*., 2000). CNGs are comprised of tetrameric proteins that are directly opened by cGMP causing the influx of Na^+ and Ca^{2+} into cells (Lucas *et al*., 2000). However, their role in regulating responses to ischaemia–reperfusion are unknown.

PKG interactions with mitochondria

As mentioned previously, cardioprotection elicited by NO and the natriuretic peptides has a mK_{ATP} channel opening component. However, evidence for direct interaction of PKG with native cardiac mK_{ATP} channels localized in the inner mitochondrial membrane remains ambiguous, because cytosolic PKG cannot migrate to this compartment (Costa *et al*., 2005). Costa *et al*. (2005) have provided evidence for PKG-induced opening of mK_{ATP} channels. Using light scattering and respiration-measurement techniques to assess mK_{ATP} channel activity, they found purified PKG-mimicked diazoxide and cromakalim-induced opening of mK_{ATP} in heart, liver and brain mitochondrial homogenates. This effect was blocked by 5-HD, tetraphenylphosphonium, glibenclamide, the PKG inhibitor KT5823, and the PKC inhibitors chelerythrine, Ro318220 and PKC-ε antagonist peptide. The authors concluded that PKG indirectly activates PKC-ε lying in the intermembrane space of the mitochondria, presumably via phosphorylation of intermediary protein(s) in the signal cascade, which in turn causes opening of the mK_{ATP} channel (Costa *et al*., 2005). This signal transduction may be in part responsible for eliciting ischaemic preconditioning. A later study by the same authors postulates that there are two distinct mitochondrial

PKC-ε subtypes, one that regulates mK_{ATP} channel opening (PKC-ε1) and the another that negatively regulates mitochondrial permeability transition (PKC-ε2; see below and Figure 2) (Costa *et al*., 2006). A major consequence of mK_{ATP} channel opening is inhibition of the electron transport chain leading to ROS generation (Andruhkiv *et al*., 2006). An evolving paradigm in cardioprotective signalling is that ROS generated as a consequence of mK_{ATP} channel opening serve an obligatory role as signalling intermediates, probably causing the activation of redox-sensitive kinases such as p42/p44 MAPK and PKC-ε (see Figure 2) (Yellon and Downey, 2003; Xu *et al*., 2004).

Mitochondrial permeability transition pore (mPTP) formation is thought to be a key event that occurs during reperfusion, leading to uncoupling of the mitochondria, loss of ionic homeostasis and cell death by necrosis (and under some conditions, by apoptosis) (Di Lisa *et al*., 2001; Halestrap *et al*., 2004). The ionic, pH and redox conditions that accompany reperfusion favour mPTP opening (Gateau-Roesch *et al*., 2006) and there is persuasive evidence that inhibition of mPTP opening preserves cell viability at reperfusion. There is no evidence that cGMP/PKG directly inhibits mPTP opening during early reperfusion in myocardium. However, cGMP-induced inhibition of mPTP formation in rat brain mitochondria extracted from reoxygenated astrocytes has been demonstrated, and was shown to be PKG-dependent (Takuma *et al*., 2001). This led Hausenloy and Yellon (2006) in their seminal review on survival kinases to propose that PKG may mediate mPTP inhibition at the time of myocardial reperfusion, in the preconditioned and postconditioned myocardium. Moreover, as illustrated in Figure 2, cGMP/PKG signalling may inhibit mPTP opening indirectly through attenuated Ca^{2+} overload and the phosphorylation of distal kinases that are known to modify mPTP opening.

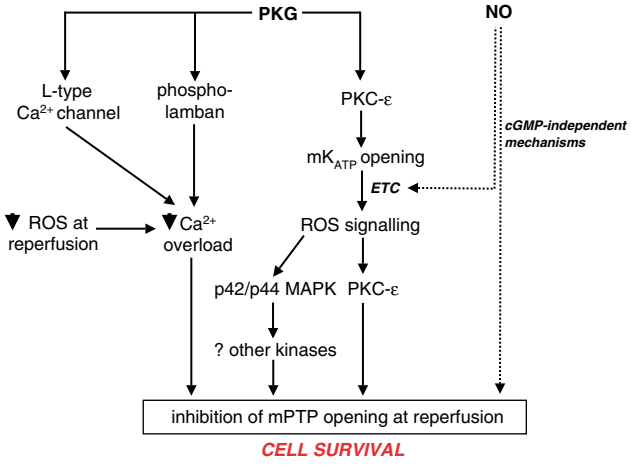

Figure 2 Putative major cardioprotective signal transduction cascades downstream of cGMP-dependent protein kinase (PKG). This general schematic integrates, in simplified form, major strands of evidence from studies in which acute protection (enhanced cell survival or infarct size limitation) has been assessed. These mechanisms may not be relevant to protection against arrhythmia development and they have not been comprehensively explored in delayed protection. A major determinant of the mode and extent of cell death is proposed to be opening of the mitochondrial permeability transition pore (mPTP) in early reperfusion. Both classical preconditioning and postconditioning, and pharmacological interventions that mimic these protective interventions, are postulated to inhibit transition pore opening at reperfusion. The cyclic guanosine 3′,5′-monophosphate/cGMP-dependent protein kinase (cGMP/PKG) pathway may influence transition pore opening indirectly in several ways. Activated-PKG phosphorylates multiple proteins associated with intracellular Ca^{2+} handling, including phospholamban and the L-type Ca^{2+} channel, attenuating intracellular Ca^{2+} load. The decrease in Ca^{2+} overload may be further enhanced by reduced production of reactive oxygen species (ROS) at reperfusion by unknown mechanisms in the protected myocardium. PKG also signals, via protein kinase C (PKC)-ε, to cause opening of the mitochondrial K_{ATP} channel (mK_{ATP}) leading to ROS generation. ROS act as signalling intermediates, activating distal kinases including p42/p44 mitogen-activated protein kinase (MAPK) and PKC-ε. Other kinases may be phosphorylated downstream of p42/p44 MAPK and PKC. It is noteworthy that nitric oxide (NO) may also directly inhibit mPTP opening and may also inhibit the electron transport chain (ETC) by mechanisms that are independent of soluble guanylate cyclase (sGC) activation and cyclic guanosine 3′,5′-monophosphate (cGMP) accumulation.

PKG and apoptosis

Apoptosis is an active (energy dependent) process of ordered cell death, characterized by cell shrinkage and membrane blebbing, chromatin condensation, DNA fragmentation and cell fragmentation, leading to the formation of apoptotic bodies and phagocytosis (Pollack and Leeuwenburgh, 2001; Suematsu *et al*., 2001; Razavi *et al*., 2005). Studies have shown NO and the natriuretic peptides to be equivocal in both inducing and inhibiting apoptosis in a variety of mammalian cell types (Shimojo *et al*., 1999; Stefanelli *et al*., 1999; Suenobu *et al*., 1999; Rabkin and Kong, 2000; Taimor *et al*., 2000; Czarnowska *et al*., 2001; Fiscus *et al*., 2002; Uchiyama *et al*., 2002; Maejima *et al*., 2003, 2005; Kato *et al*., 2005; Zhu *et al*., 2005). NO and ANP induced apoptosis in rat vascular endothelial cells in a concentration-dependent manner (Suenobu *et al*., 1999). Using SNAP and (+/−)-(E)-4-ethyl-2-[(E)-hydroxyimino]-5-nitro-3-hexeneamide (NOR3), Shimojo *et al*. (1999) demonstrated the pro-apoptotic effect of the NO/cGMP/PKG pathway in cultured neonatal rat cardiomyocytes. This has been corroborated in similar studies using rat cardiomyocytes (Rabkin and Kong, 2000; Taimor *et al*., 2000; Uchiyama *et al*., 2002). Other conflicting studies have illustrated the anti-apoptotic effects of NO and the natriuretic peptides: SNAP inhibited cardiomyocyte apoptosis via modulating cyclin A-associated kinase activity (Maejima *et al*., 2003); ANP inhibited cardiomyocyte apoptosis by indirectly inducing nuclear accumulation of zyxin (regulator of actin filament assembly) and Akt (Kato *et al*., 2005).

Interplay between tumour necrosis factor-α (TNF-α) and the TNF death receptors (TNFR1) triggers NO-induced apoptosis in cardiomyocytes (Song *et al*., 2000). Basal levels of TNF-α do not permit apoptosis in cardiomyocytes, but under inflammatory conditions, that is during ischaemia–reperfusion, TNF-α increases iNOS expression initiating the apoptotic process (Song *et al*., 2000; Razavi *et al*., 2005). Whether there is a cGMP/PKG component to this mechanism remains to be elucidated. The anti-apoptotic effects of NO mediated through the cGMP/PKG signalling cascade could be due to increased expression of the anti-apoptotic

protein bcl-2, and inhibition of mPTP formation (Razavi *et al*., 2005). Furthermore, the NO and the natriuretic peptides may prevent cardiomyocyte apoptosis via cGMP/PKG-dependent inhibition of intracellular calcium overload, as mentioned earlier.

Perspectives and conclusion

A large body of experimental evidence points to cGMP accumulation as a tractable pharmacological target for attenuating ischaemia–reperfusion injury. Many of the possible injury-limiting mechanisms could be related to activation of PKG, including SR regulation of Ca^{2+} homeostasis, opening of the mK_{ATP} channel, and recruitment of the anti-apoptotic protein bcl-2. Activation of the cGMP/PKG pathway as a possible therapeutic means of attenuating ischaemia–reperfusion injury holds several attractions. First, several endogenous autacoid mediators that are recognized to be cardioprotective signal directly or indirectly through cGMP accumulation. These autacoids include NO and the natriuretic peptides, but other mediators such as bradykinin, insulin and adrenomedullin are also known to exert their infarct-limiting effects via NO generation and subsequent cGMP accumulation (see Figure 1) (Baxter and Ebrahim, 2002; Oldenburg *et al*., 2004; Hamid and Baxter, 2005, 2006; Abdallah *et al*., 2006; Hamid *et al*., 2007b). Second, all of the autacoids that enhance GMP accumulation and several drugs that do so, either directly or indirectly, appear to exert a protective action when administered exogenously during early reperfusion. Thus, exogenously administered natriuretic peptides, adrenomedullin and bradykinin, targeted during the early reperfusion period, limit infarct size. NO donor compounds and L-arginine (the physiological NO precursor) may have the same potential. This point is of great relevance to therapeutic application since the majority of MIs are unheralded, with patients presenting after ischaemia onset. Until the relatively recent discovery of postconditioning and the recognition that signalling pathways activated in early reperfusion have the potential to salvage reperfused myocardium, the translation of any experimental cardioprotective agents into the clinical arena seemed futile. The basic science now tends to support the targeted dosing with relevant cardioprotective agents as an adjunct to reperfusion. Third, the potential for activation of the cGMP/PKG pathway to regulate long-term cardioprotective adaptation through a delayed preconditioning-like mechanism could underpin the development of such drugs for prophylactic use in patients with diagnosed coronary disease and therefore at high risk of ischaemia–reperfusion injury. Fourth, the limited tissue distribution of cGMP-specific PDEs such as PDE5 may favour the clinical use of existing PDE5 inhibitors in the acute management of ischaemia–reperfusion, and the development of 'cardioselective' PDE5 inhibitors for long-term treatment.

Considerable obstacles to the further exploration and development of the cGMP/PKG pathway remain. To a large degree, the major obstacle is ignorance of the basic biology of the pathway. Although cGMP and PKG have been known for several decades, our knowledge of how these mediators regulate cardiac function and how they respond during ischaemia–reperfusion is only now becoming clear. The complexities of cGMP regulation extend far beyond its production and degradation, since it is likely that subcellular pools (compartments) of cGMP may be determined by the localization of soluble and particulate guanylyl cyclases, and cGMP-sensitive PDEs. Hence, global measures of tissue cGMP concentration in experimental models may not adequately explain mechanisms of action. Similarly, the precise role played by PKG is at present difficult to determine. Pharmacological inhibitors of PKG have limitations associated with selectivity, as well as the economic hurdles of using these compounds at effective concentrations in intact heart preparations or for *in vivo* studies. Although a non-conditional PKG-I knockout mouse has been developed (Pfeifer *et al*., 1998), the gastro-intestinal and cardiovascular phenotypes of these animals are highly abnormal, resulting in systemic hypertension, nutritional disorder and 80% mortality within 8 weeks. A cardiac conditional mutation could prove to be a most valuable experimental tool. With regard to the role of cGMP/PKG signalling in cardioprotective signalling, interactions with the other kinase cascades are unclear at present. Although PI3K/Akt activation leads to eNOS activation and NO generation, with subsequent activation of sGC and cGMP elevation and PKG activation, the generation of ROS as a consequence of PKG stimulation of mK_{ATP} could plausibly lead to the activation of distal kinases. What these distal kinases are and how they relate to limitation of cell injury, either during ischaemia or reperfusion, is unknown. Moreover, the activity of cGMP may not be easily disentangled from that of cAMP, as the cGMP-sensitive PDE3 can lead to elevation of cAMP (Kojda and Kottenberg, 1999). Studies have shown exogenous NO and the natriuretic peptides to both induce and inhibit apoptosis in a variety of mammalian cell types: whether these differential effects are related to cGMP concentration and/or the extent and timing of PKG activation, or due to cell type and experimental conditions is unclear.

Patients at risk of ischaemia–reperfusion injury often have co-morbidities contributing to coronary artery disease. These include hypertension (with or without cardiac hypertrophy), dyslipidaemias and diabetes. So far, the extent of experimental investigations of cardioprotection in models representing these co-morbidities is very limited. However, it has been proposed that ignorance of the ways in which these disease states in man can modify cardioprotective signalling pathways may constitute a major obstacle to therapeutic translation of basic science studies (Ferdinandy *et al*., 1998; Kloner *et al*., 2002). For example, it has been known for more than a decade that hyperlipidaemic rats have an imparied preconditioning response (Szilvassy *et al*., 1995). Although the nature of the biochemical defect is unknown, the loss of protection appears to be through a direct effect of hyperlidaemia on the myocardium. Recent work suggests that the cGMP/PKG pathway may be severely impaired in hyperlipidaemia since 8-bromo-cGMP, SNAP and BNP were all ineffective in eliciting infarct size limitation in hearts from chronically hyperlipidaemic rats, even though the agents were all protective in normal hearts (Giricz *et al*., 2007). It is important to note that clinical experience with some of the

agents discussed here raises questions about the safety and tolerability of highly vaso-active drugs in the setting of acute ischaemia–reperfusion in patients. For example, the tendency for the NO donors (notably organic nitrates) to induce the 'coronary steal' phenomenon underlies their unsuitability for the treatment of MI (Abrams, 1996).

In conclusion, intracellular accumulation of cGMP and activation of PKG is associated with attenuation of the pathologies associated with ischaemia–reperfusion injury, notably infarct development. The vast majority of studies with pharmacological modulators of cGMP point towards intracellular cGMP as being a protective mediator. PKG, activated by elevated cGMP, seems to be the most likely candidate for effecting the cardioprotective actions of cGMP but direct evidence implicating PKG as a survival kinase is limited at present. However, there are several plausible downstream targets of PKG that could be responsible for mediating NO and natriuretic peptide-induced cardioprotection. These include ion channels regulating Ca^{2+} homeostasis, the SR and the mK_{ATP} channel: the latter appears to be pivotal in the protection provided by most endogenous mediators of preconditioning and postconditioning. Thus, new experimental evidence and the re-interpretation of old work in the light of current knowledge strongly supports a pivotal role of the cGMP/PKG pathway as a survival signal in ischaemia–reperfusion. The challenges of enhancing this knowledge to the point where it can be translated from the bench to the clinic remain substantial. However, with so many experimental agents, including a range of cardiac autacoids, signalling via cGMP, there is a case for cautious optimism that this pathway holds promise for therapeutic development.

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Conflict of interest

The authors state no conflict of interest.

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