

REVIEW

The cGMP/PKG pathway as a common mediator of cardioprotection: translatability and mechanism

Javier Inerte and David Garcia-Dorado

Cardiology Department, Vall d'Hebron University Hospital and Research Institute, Universitat Autònoma de Barcelona, Barcelona, Spain

Correspondence

Dr David Garcia-Dorado, Servicio de Cardiología, Hospital Universitari Vall d'Hebron, Passeig Vall d'Hebron, 119-129, 08035 Barcelona, Spain. E-mail: dgdrorado@vhebron.net

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Cardiomyocyte cell death occurring during myocardial reperfusion (reperfusion injury) contributes to final infarct size after transient coronary occlusion. Different interrelated mechanisms of reperfusion injury have been identified, including alterations in cytosolic Ca^{2+} handling, sarcoplasmic reticulum-mediated Ca^{2+} oscillations and hypercontracture, proteolysis secondary to calpain activation and mitochondrial permeability transition. All these mechanisms occur during the initial minutes of reperfusion and are inhibited by intracellular acidosis. The cGMP/PKG pathway modulates the rate of recovery of intracellular pH, but has also direct effect on Ca^{2+} oscillations and mitochondrial permeability transition. The cGMP/PKG pathway is depressed in cardiomyocytes by ischaemia/reperfusion and preserved by ischaemic postconditioning, which importantly contributes to postconditioning protection. The present article reviews the mechanisms and consequences of the effect of ischaemic postconditioning on the cGMP/PKG pathway, the different pharmacological strategies aimed to stimulate it during myocardial reperfusion and the evidence, limitations and promise of translation of these strategies to the clinical practice. Overall, the preclinical and clinical evidence suggests that modulation of the cGMP/PKG pathway may be a therapeutic target in the context of myocardial infarction.

LINKED ARTICLES

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

Abbreviations

AMI, acute myocardial infarction; ANP, atrial natriuretic peptide; BH₄, tetrahydrobiopterin; CaMK, Ca^{2+} /calmodulin-dependent PK; eNOS, endothelial NOS; GIK, glucose-insulin-potassium; GLP-1, glucagon-like peptide-1; GSK3 β , glycogen synthase kinase-3 β ; mPTP, mitochondrial permeability transition pore; NHE, Na⁺/H⁺ exchanger; NP, natriuretic peptide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PCI, percutaneous coronary intervention; pGC, particulate GC; pHi, intracellular pH; PLB, phospholamban; PoCo, ischaemic postconditioning; PreC, ischaemic preconditioning; RIC, remote ischaemic conditioning; RISK, reperfusion injury salvage kinases; ROS, reactive oxygen species; SERCA, sarcoplasmic reticulum Ca^{2+} -ATPase; sGC, soluble GC; SR, sarcoplasmic reticulum; STEMI, ST-segment elevation myocardial infarction

Tables of Links

TARGETS		LIGANDS	
Enzymes^a	PI3K	Adenosine	GLP-1, glucagon-like peptide-1
Akt	PKA	ANP, atrial natriuretic peptide	Insulin
CaMKII, Ca ²⁺ /calmodulin-dependent PK	PKG	Ataciguat	L-arginine
eNOS, endothelial NOS	SERCA	BH4, tetrahydrobiopterin	NO, nitric oxide
ERK1/2	sGC, soluble GC	Brain natriuretic peptide	ODQ,[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
GSK3 β , glycogen synthase kinase-3 β	Transporters^b	cAMP	Riociguat
PDE2	Na/Ca exchanger	cGMP	Sildenafil
PDE5	NHE, Na ⁺ /H ⁺ + exchanger	Cinaciguat	Vardenafil
		Exenatide	YC-1

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

Ischaemia/reperfusion injury

Cardiomyocyte death is the main cause of the mortality and morbidity in patients with ischaemic heart disease, the leading cause of death in the global world population. It occurs mainly during the acute coronary syndrome, and in particular during myocardial infarction with ST-segment elevation, for which the main treatment is prompt reperfusion. There is solid evidence supporting that part of cell death, secondary to transient coronary occlusion, occurs upon restoration of coronary blood flow, a phenomenon known as reperfusion injury (Piper *et al.*, 1998; Yellon and Hausenloy, 2007).

The mechanisms responsible for cell death during myocardial reperfusion are complex but progressively better known, and have been reviewed recently (Garcia-Dorado *et al.*, 2012; 2014a). Restoration of coronary blood flow triggers the synthesis of ATP and Ca²⁺ uptake by the sarcoplasmic reticulum (SR) through the sarcoplasmic reticulum Ca²⁺-ATPase (SERCA), exceeding its capacity and resulting in the abrupt release of Ca²⁺ (through ryanodine receptors 2), followed by reuptake and resulting in cyclic Ca²⁺ oscillations. These oscillations can cause arrhythmias, hypercontracture and sarcolemmal rupture (Piper *et al.*, 2006). During reperfusion, Ca²⁺ influx from the extracellular space occurs mainly via the Na⁺/Ca²⁺ exchanger and the increase in cytosolic Ca²⁺ activates calpain proteolysis, which damages the cytoskeleton and alters the function of the Na⁺ pump (Inserte *et al.*, 2005), aggravating Na⁺ and, secondarily, Ca²⁺ overload. On the other hand, the production of free radicals together with Ca²⁺ overload induces mitochondrial permeabilization and energy failure leading to cell death (Penna *et al.*, 2013). Due to the preferential communication between mitochondria and SR, Ca²⁺ oscillations can cause mitochondrial permeabilization and *vice versa* (Ruiz-Meana *et al.*, 2007; Abdallah *et al.*, 2011). It is important to note that proteolytic activation, mitochondrial permeabilization and hypercontracture are dependent on intracellular pH (pHi) and do not occur until

intracellular acidosis is corrected during the initial minutes of reperfusion (Inserte *et al.*, 2011a).

It is now clear that cAMP/cGMP-dependent PK (cGMP/PKG) signalling modulates not only the rate of pHi normalization, but many other important phenomena determining cell death or survival during myocardial reperfusion. This article will review the effects of ischaemia/reperfusion on the cGMP/PKG pathway, its role in endogenous cardioprotection by postconditioning and its potential as a pharmacological target for the prevention of reperfusion injury.

Effect of ischaemia/reperfusion on the cGMP pathway

cGMP is a ubiquitous intracellular second messenger involved in a large variety of cardiovascular processes (Yuan, 2002; Munzel *et al.*, 2003; Tsai and Kass, 2009). cGMP is produced from the purine nucleotide GTP in an enzymic reaction mediated by two different types of guanylyl cyclase (GC) that differ in their ligands and intracellular distribution (Cerra and Pellegrino, 2007) (Figure 1). One is the soluble GC (sGC), localized in the cytosol and requiring binding of endogenous NO to its haem moiety for cGMP synthesis. The other is the group of particulate GC (pGC) that is the integral proteins of the plasmatic membrane specifically activated by natriuretic peptides (NPs). A natriuretic receptor (natriuretic peptide receptor-C), without GC activity, binds to a G_i protein eliciting activation of endothelial NOS (eNOS) (Costa *et al.*, 2006). The cellular activity of cGMP is controlled through its highly regulated hydrolytic degradation by PDEs (Bender and Beavo, 2006) that differ in their affinity and specificity for cAMP and cGMP, and in their distribution, not only across tissues but also among the different subcellular compartments of cardiomyocytes (Zaccolo and Movsesian, 2007; Rao and Xi, 2009; Francis *et al.*, 2011). The net effect of this system of distribution of PDE activity is that the concentration of cGMP and cAMP varies within different intracellu-

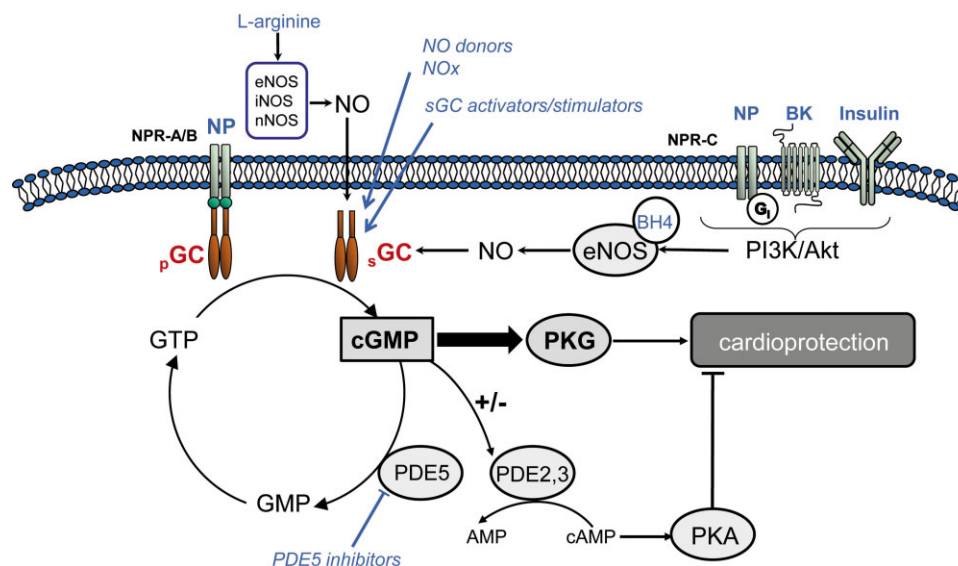


Figure 1

Schematic representation of the cGMP/PKG pathway. cGMP is synthesized by activation of the NO/sGC and the NP/pGC pathways and regulated by compartmentalized PDEs. Agonists of G_i -coupled receptors stimulate the eNOS/sGC/PKG axis via activation of PI3K/Akt. The main cGMP actions are not only mediated by PKG but also by regulation of cAMP-mediated effects through modulation of PDE2 and PDE3 activities. The different pharmacological strategies that have been used to stimulate the cGMP/PKG pathway are shown in blue. BK, bradykinin; NOx, nitrites/nitrates.

lar compartments in response to pGC and sGC stimulation (Castro *et al.*, 2010). The primary cGMP mediator in the cardiovascular system is the cGMP-dependent PKI (PKG), which acts by phosphorylating target proteins and, indirectly, the cAMP-dependent PK (PKA) via cGMP-regulated PDE (Zhang and Kass, 2011).

Ischaemia/reperfusion has dramatic effects on the basal myocardial content of cGMP and the ability of cardiomyocytes to respond to stimulators of cGMP synthesis. In the isolated heart model, myocardial cGMP levels increase during the first 10–25 min of ischaemia (Depre and Hue, 1994; Lochner *et al.*, 1998) and acutely decrease thereafter (Nesher *et al.*, 1977; Inserte *et al.*, 2000). Reduced myocardial cGMP content has been described in the *in situ* rat, rabbit and pig heart subjected to transient ischaemia (Yamaguchi *et al.*, 1997; Hoshida *et al.*, 1999; Padilla *et al.*, 2001).

Several factors can modify cGMP synthesis during ischaemia and reperfusion. Energetic depletion could reduce the availability of GTP, the substrate for GC, or affect GC activity by modification in its phosphorylation state (Lucas *et al.*, 2000). However, we have observed no correlation between intracellular ATP content and cGMP synthesis stimulated by NO donors or pGC agonists in isolated cardiomyocytes (Agullo *et al.*, 2003). In agreement with these results, it has been reported that a depletion of GTP to less than 10% of the initial levels did not decrease, but substantially increased cGMP synthesis after stimulation with a NO donor (Geisbuhler and Schwager, 1996). Further studies are needed to understand this absence of effect of ATP concentration on cGMP in cardiomyocytes.

Acidosis has been shown to be critical for cGMP synthesis. Results from our group demonstrated that pHi values close to 6.4 induce a profound depressant effect on pGC in cultures of

cardiomyocytes and endothelial cells and on sGC in endothelial cells (Agullo *et al.*, 2003). This study suggests that in situations causing intracellular acidification, as ischaemia, cardiomyocyte cGMP synthesis is largely dependent on NO stimulation of sGC. However, oxidative stress associated with ischaemia/reperfusion may decrease sGC activity by direct oxidation of its haem group (Stasch *et al.*, 2006; Zhou *et al.*, 2008; Derbyshire and Marletta, 2012). In addition, reduced NO bioavailability during reperfusion is a consequence of eNOS uncoupling by oxidation of its cofactor tetrahydrobiopterin (BH4) (Vasquez-Vivar *et al.*, 2002; Chen *et al.*, 2010).

cGMP/PKG pathway as a mediator of endogenous cardioprotection

Repeated non-lethal episodes of ischaemia/reperfusion activate endogenous protective mechanisms that profoundly reduce the infarct caused by a more sustained ischaemic insult. This concept of endogenous cardioprotection was initially described for ischaemic preconditioning (Murry *et al.*, 1986), which is a pretreatment by definition and therefore cannot be used in the setting of acute myocardial infarction (AMI). Subsequently, it has been expanded to the more clinically applicable paradigms of postconditioning (ischaemic postconditioning; PoCo) and remote conditioning (Kharbanda *et al.*, 2002; Zhao *et al.*, 2003; Kerendi *et al.*, 2005).

Postconditioning, consisting in brief episodes of coronary artery reocclusion and reflow applied at the moment of myocardial reperfusion, is by far the cardioprotective strategy that has been tested in more proof-of-concept clinical trials, and

the first demonstrating that it is possible to reduce infarct size in patients with ST-segment elevation myocardial infarction (STEMI) using treatments applied at the time of reperfusion. In most of these small clinical trials, cycles ranging from 30 to 90 s of angioplasty balloon inflation and deflation have been shown to improve both, early markers of infarct size and contractile function up to 1 year of infarction (Staat *et al.*, 2005; Thibault *et al.*, 2008; Ovize *et al.*, 2010). There are, however, studies that do not confirm protection by PoCo, questioning the utility of this phenomenon to limit reperfusion injury in clinical practice (Sorensson *et al.*, 2010; Freixa *et al.*, 2012; Tarantini *et al.*, 2012). A recent multicentre, prospective, randomized trial with the largest series reported so far [700 patients with STEMI undergoing primary percutaneous coronary intervention (PCI)] failed to demonstrate a benefit of a PoCo protocol consisting in four cycles of 1 min low-pressure balloon inflation separated by 1 min of reflow (Hahn *et al.*, 2013). The results of a large-scale trial (DANAMI-3, NCT01435408) that is currently underway to determine whether PoCo can reduce the combined end-point of cardiac death, re-infarction and heart failure at 3 years will provide more definitive information about the clinical usefulness of PoCo and the conditions that determine its effectiveness.

Although different confounding factors, including co-morbidities, co-medications and interventional procedures, have been proposed to explain the coexistence of positive and negative clinical studies (Heusch, 2012; Hausenloy *et al.*, 2013), it is important to note that not all preclinical studies have consistently shown a benefit of PoCo. The reasons for these discrepancies lie primarily in the PoCo algorithm used, the effectiveness of which varies depending on the species and experimental preparation (Skyschally *et al.*, 2009a). The critical factor determining the threshold for triggering PoCo protection is the ability of the PoCo algorithm to delay pHi normalization at the onset of reperfusion (Cohen *et al.*, 2007; Fujita *et al.*, 2007; Inserte *et al.*, 2009). Measurement of pHi kinetics by NMR spectroscopy in rat hearts showed that only those PoCo protocols that prolong acidosis during reperfusion for at least 3 min are protective and that there is a close correlation between the delay in pHi recovery and the extent of cell death (Inserte *et al.*, 2009). As it is not possible to accurately measure pHi in patients undergoing PCI, we do not know whether the PoCo algorithms used in clinical trials with negative results were adequate to ensure a slow pHi correction at the time of reflow.

The cardioprotective effect of prolongation of intracellular acidosis during the first minutes of reperfusion has been solidly demonstrated in different models (Kaplan *et al.*, 1995; Ohashi *et al.*, 1996; Preckel *et al.*, 1998; Inserte *et al.*, 2008). As detailed in a recent review, acidosis inhibits many mechanisms involved in reperfusion injury including Ca^{2+} overload and myofibrillar contractility that causes Ca^{2+} -dependent hypercontracture, mitochondrial permeability transition pore (mPTP) formation, calpain activation and propagation of cell death through gap junctions (Inserte *et al.*, 2011a). Preventing cell death during acidosis allows for activation of endogenous mechanisms of protection and/or normalization of Ca^{2+} homeostasis that would prolong protection once the pHi has been normalized. On the basis of these findings, we propose that the relative timing of the correction of intracel-

lular Ca^{2+} levels and pHi during the first minutes of reperfusion will determine cell death (recovery of pHi occurs before that of Ca^{2+}) or survival (recovery of Ca^{2+} control occurs before pHi normalization).

The mechanism by which PoCo delays the normalization of pHi during early reperfusion involves not only reduced lactate washout caused by intermittent reflow but also the activation of the cGMP/PKG pathway (Inserte *et al.*, 2011b). Blockade of cGMP/PKG pathway by the addition of the sGC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) or the PKG inhibitor KT5823 during reperfusion accelerated pHi recovery measured by NMR spectroscopy and abolished PoCo protection in isolated hearts. The effect of PoCo on pHi appears to be mediated by an inhibitory effect of PKG on the Na^+/H^+ exchanger (NHE) (Inserte *et al.*, 2011b).

Activation of cGMP/PKG pathway by ischaemic conditioning

There is good evidence demonstrating that activation of cGMP/PKG pathway is essential for the protective effects of PoCo (Yang *et al.*, 2004; Penna *et al.*, 2006; Inserte *et al.*, 2011b). As in ischaemic preconditioning (PreC), activation of sGC and PKG by PoCo has been proposed to be dependent on the PI3K/Akt pathway. Hausenloy *et al.* found that activation of the prosurvival kinases PI3K/Akt and ERK1/2 during the first minutes of reperfusion, which they termed the reperfusion injury salvage kinases (RISK) pathway, is required for PoCo protection (Hausenloy *et al.*, 2005). However, the causal role of RISK in the protection induced by PoCo has been questioned by more recent data. Different studies performed in the *in situ* pig model and the rat perfused heart demonstrated that PoCo was still effective after pharmacological blockade of RISK and that increased RISK phosphorylation was a consequence but not a cause of the reduction in cell death induced by PoCo (Skyschally *et al.*, 2009b; Inserte *et al.*, 2013). Moreover, the observation that blockade of NOS, sGC and PKG, but not PI3K, abolished the cardioprotective effects of PoCo, confirmed that activation of the cGMP/PKG pathway depends on NOS but is independent of PI3K/Akt signalling (Inserte *et al.*, 2013).

Recently, an alternative mechanism, summarized in Figure 2, for increased cGMP/PKG signalling has been proposed as well. Different studies have observed that PoCo attenuates reactive oxygen species (ROS) generation at reperfusion, probably by limiting the delivery of oxygen during the controlled reperfusion (Halkos *et al.*, 2004; Kin *et al.*, 2004; Fan *et al.*, 2011). Our laboratory reported similar results in an *ex vivo* rat heart model and demonstrated that the resulted attenuation of oxidative stress by PoCo reduces eNOS uncoupling by preserving cytosolic BH4 levels and results in increased NO-dependent activation of cGMP/PKG pathway (Inserte *et al.*, 2013).

Mechanism of cGMP-mediated cardioprotection

Despite the existing evidence supporting that the cGMP/PKG pathway is required for PoCo protection, only few studies have identified target proteins phosphorylated by PKG that are involved in the molecular mechanisms of cardioprotection. Activation of PKG has been suggested to inhibit mPTP

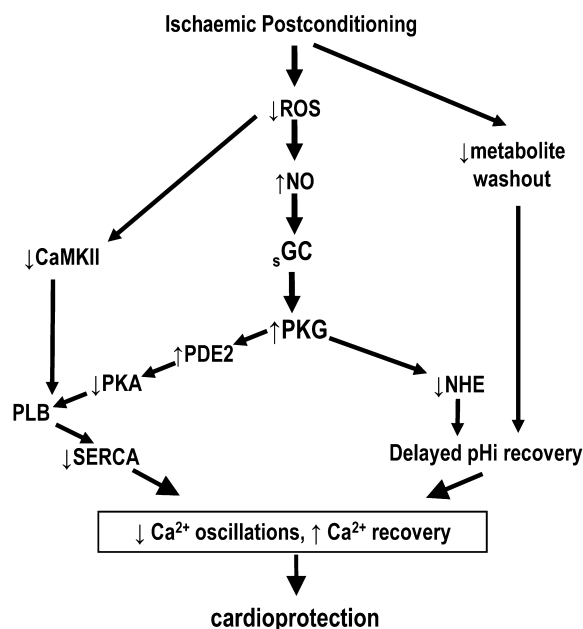


Figure 2

Scheme showing the proposed mechanisms by which cGMP/PKG pathway participates in the cardioprotective effects of ischaemic postconditioning (PoCo). PoCo attenuates the burst of O_2^- generated at the onset of reperfusion, reducing oxidative stress which limits the oxidation of BH4 and increases eNOS activity. The resulting activation of the cGMP/PKG pathway contributes to PoCo protection at least in part by delaying normalization of pHi during reperfusion via PKG-dependent inhibition of NHE. In addition, PoCo delays PLB phosphorylation at the onset of reperfusion by inhibiting PKA and CaMKII. The resulting transient inhibition of SERCA prevents oscillations Ca^{2+} and favours Ca^{2+} extrusion through Na^+/Ca^{2+} exchanger.

by phosphorylation of glycogen synthase kinase-3 β (GSK3 β) (Juhászova *et al.*, 2004; Gomez *et al.*, 2008). However, double knockin mice lacking the GSK3 β phosphorylation sites that result in kinase inactivation could still be protected by both PreC and PoCo (Nishino *et al.*, 2008), demonstrating that the inhibition of GSK3 β is unlikely to serve as a downstream mediator of the cardioprotection mediated by cGMP/PKG activation. More recently, the notion that GSK3 β phosphorylation does not play a causal role in PoCo protection has been further supported by data from the *in situ* pig model and the *ex vivo* rat heart (Skyschally *et al.*, 2009b; Inserte *et al.*, 2013).

In addition to its contribution to prolong acidosis at the onset of reperfusion by inhibition of NHE, it is proposed that PKG modulates phospholamban (PLB) phosphorylation and, therefore, SR Ca^{2+} entry through SERCA (Abdallah *et al.*, 2005). At reperfusion, PLB is rapidly phosphorylated by PKA at Ser¹⁶ and Ca^{2+} /calmodulin-dependent PK (CaMK) at Thr¹⁷ resulting in its dissociation from SERCA and activation of the pump (Vila-Petroff *et al.*, 2009), favouring the formation of SR-driven Ca^{2+} oscillations that cause reperfusion-induced hypercontracture and mPTP opening (Siegmund *et al.*, 1997). In a recent study, we have demonstrated that PoCo reduces SERCA activity during the first minutes of reperfusion by delaying PLB phosphorylation through activation of PKG and

inhibition of PKA and CaMKII. Additionally, our study suggests that cGMP/PKG-dependent activation of PDE2 is responsible for the inhibition of PKA while the reduced activity of CaMKII could be the result of PoCo-dependent attenuation of Ca^{2+} overload and ROS production during the first minutes of reperfusion (Erickson *et al.*, 2011). The resulting blockade of SERCA activity during the first minutes of reperfusion attenuated the development of hypercontracture and cell death by preventing Ca^{2+} oscillations and allowed for Ca^{2+} normalization through the Na^+/Ca^{2+} exchanger and for the activation of endogenous protective signalling pathways that ensure long-term cardioprotection (Inserte *et al.*, 2014).

Prolongation of acidosis by PoCo increases S-nitrosylated protein levels (Penna *et al.*, 2011) and, recently, the selective sGC inhibitor ODQ (Methner *et al.*, 2013; Tong *et al.*, 2014) and the cardiomyocyte-specific ablation of the PKGI gene (Methner *et al.*, 2013) failed to abolish PoCo-induced protection in isolated mouse hearts, suggesting that NO-mediated S-nitrosylation rather than the sGC/PKG pathway plays a key role in the cardioprotective effects of PoCo. However, although ODQ, which inhibits sGC by binding to its haem group in a NO-competitive manner (Schrammel *et al.*, 1996), is commonly used to discriminate cGMP-dependent and cGMP-independent effects of NO, it has been demonstrated that persistence of NO-induced effects in the presence of ODQ does not necessarily prove cGMP independence (Lies *et al.*, 2013). In addition, it cannot be ruled out that the persistence of protection in mice lacking PKGI in cardiomyocytes is a consequence of compensatory mechanisms including the effect of other PKG isoforms or the contribution of PKGI from other cardiac cell types.

Activation of cGMP/PKG pathway in remote ischaemic conditioning

A major limitation of PoCo is that it can only be performed during the process of PCI, reducing its target patient population but also increasing the risk of complications during the procedure. In recent years, it has been reported that induction of intermittent non-lethal ischaemia in a remote organ or limb protects the myocardium against the detrimental effects of a sustained lethal episode of ischaemia. This stimulus known as remote ischaemic conditioning (RIC) can be applied before myocardial ischaemia, during myocardial ischaemia or in the first few minutes of reperfusion (Andreka *et al.*, 2007; Schmidt *et al.*, 2007). RIC has multiple advantages over PoCo including the fact that it can be easily applied in the ambulance to all patients with STEMI that receive reperfusion treatment and is extremely safe (Botker *et al.*, 2010). By contrast, the mechanisms by which RIC exerts its protective effect are much less known. The pathway linking the remote organ, on which the stimulus is applied, to the heart remains unclear and has been attributed to neural and/or hormonal pathways. Recently, Donato *et al.* described that remote preconditioning activates a neural afferent pathway and the cardioprotective signal reaches the heart through the vagus nerve (efferent pathway) and releases ACh that activates the endogenous signal cascade through muscarinic receptors (Donato *et al.*, 2013), which can result in the stimulation of eNOS and increased production of cGMP (Massion and Balligand, 2003; Downey *et al.*, 2007). Other studies provide evidence suggesting the involvement

of cGMP/PKG pathway via activation of the PI3K/Akt-dependent signalling by released humoral factors (Breivik *et al.*, 2010; Xin *et al.*, 2010). Hausenloy *et al.* reported that in the *in situ* pig model, activation of the PI3K/Akt pathway at the time of reperfusion is required for the cardioprotective effects of remote preconditioning but not of remote perconditioning (Hausenloy *et al.*, 2012).

Myocardial cardioprotection by pharmacological modulation of the cGMP/PKG pathway

There is extensive literature demonstrating that preservation of the cGMP/PKG pathway during myocardial reperfusion by using pharmacological strategies directed to either increase cGMP synthesis or reduce its degradation attenuates cardiomyocyte death occurring with reperfusion (Bice *et al.*, 2013). However, it is important to note that the experimental design used in many of these studies includes the administration of the tested drug before the ischaemic insult, a situation that does not adequately model the clinical context of a patient with ongoing AMI receiving myocardial reperfusion. Unfortunately, several of these pharmacological interventions have provided less conclusive data regarding their effectiveness to increase cGMP production and to limit ultimate infarct size when they are applied at the time of reperfusion.

This section summarizes the pharmacological strategies that have been tested in preclinical studies to stimulate the cGMP/PKG pathway and limit myocardial reperfusion injury, making emphasis in those with potential for their translation to the clinical treatment of AMI.

Stimulation of cGMP synthesis

Most of the preclinical data supporting preservation of cGMP/PKG pathway as a therapeutic strategy for protecting myocardium against reperfusion injury are based in studies that stimulate the synthesis of cGMP. The potential targets that can augment cGMP levels are divided in those acting through the NO/sGC pathway and the NPs/pGC pathway.

NO/sGC pathway

Synthesis of cGMP through sGC is modulated by NO generated by NOS using L-arginine as a substrate, O₂ and NADPH. However, in disease states associated with reduced availability of O₂ and high-energy phosphates, as occurs during ischaemia, production of NO is severely compromised (Lundberg *et al.*, 2008). L-arginine supplementation has been pursued as a potential approach for restoring NO production in reperfused myocardium. The pre-ischaemic administration of L-arginine has been found to attenuate reperfusion injury in isolated rat and *in situ* pig hearts by a mechanism dependent on the sGC (Agullo *et al.*, 1999; Padilla *et al.*, 2000). However, its administration at the onset of reperfusion failed to increase cGMP levels and protect myocardium (Agullo *et al.*, 1999). Impaired NOS activity as consequence of the burst of ROS generated during the first minutes of reperfusion may explain the lack of efficacy of L-arginine. Superoxide generated from different sources combines with NO at a very fast

rate to form peroxynitrite (ONOO⁻) ions (Beckman *et al.*, 1990). Peroxynitrite not only reduces the availability of free NO but may also act directly on the NOS haem group producing the inactivation of the enzyme (Chen *et al.*, 2010). In addition, O₂⁻ and ONOO⁻ can oxidize BH₄, an essential cofactor for NOS, resulting in NOS uncoupling and preferential production of O₂⁻ instead of NO (Xia *et al.*, 1998; Vasquez-Vivar *et al.*, 2002).

Because different studies demonstrate that BH₄ is markedly reduced during ischaemia (Dumitrescu *et al.*, 2007), the therapeutic potential of BH₄ supplementation has been analysed (Moens *et al.*, 2011). Pretreatment with BH₄ or the BH₄ precursor sepiapterin has been shown to improve endothelium-dependent vasorelaxation in isolated coronary arterioles and ventricular function in isolated rat hearts following ischaemia/reperfusion injury (Tiefenbacher *et al.*, 1996; Verma *et al.*, 2002; Yamashiro *et al.*, 2002). Our laboratory has examined the effect of BH₄ supplementation during the first minutes of reperfusion in rat isolated hearts. The results showed that exogenous BH₄ was effective in reducing NOS uncoupling and increasing NO production but was associated with no effect on O₂⁻ levels, enhanced ONOO⁻ production and failure to protect myocardium (Inserte *et al.*, 2014). This study indicates that NOS uncoupling is not a major source for O₂⁻ generated during myocardial reperfusion and support the notion that the effectiveness of those strategies directed to stimulate NOS activity is limited by the excessive O₂⁻ production and the subsequent formation of ONOO⁻, an effect that could counterbalance the potential cardioprotective actions of enhanced NOS activity.

An alternative approach to stimulate the NO/sGC pathway that would bypass NOS involves the use nitrates (nitroglycerin, isosorbide mononitrate) and nitrites (sodium nitrite, amyl nitrite) which are reduced in sequential steps by enzymatic and non-enzymic mechanisms generating NO, and synthetic NO donors which spontaneously release NO (SNAP, NOC9) (Lundberg *et al.*, 2008). The cardioprotective actions of these compounds have been examined by numerous studies and there is solid evidence supporting that their administration before ischaemia diminishes infarct size by NO-dependent, NOS-independent mechanisms (Webb *et al.*, 2004; Duranski *et al.*, 2005; Shiva *et al.*, 2007). However, the literature is inconsistent in reporting protective effects of NO donors when administered at reperfusion in animal models. Bice *et al.* demonstrated a concentration-dependent effect of NOC9, a rapid release NO donor, limiting infarct size in *ex vivo* rat hearts (Bice *et al.*, 2014), while in a study by Salloum *et al.*, nitroglycerin given at reperfusion failed to limit infarct size in an *in vivo* rabbit model (Salloum *et al.*, 2007). The NO donor SNAP in the range of 1–10 µM was not protective in *ex vivo* hearts when administered for 15 min (Burley and Baxter, 2007), but reduced infarct size when its infusion was extended to 35 min of reperfusion, suggesting that a prolonged time of infusion is necessary to maintain the cardioprotective signalling (Cohen *et al.*, 2010). However, an alternative explanation for this inconsistent effectiveness of NO donors is related to the oxidative stress caused by reperfusion. First, as it occurs with those strategies aimed to stimulate NOS, NO donors can favour the deleterious effects resulting from the formation of ONOO⁻ when they are administered at the time of the burst of ROS generated at

reperfusion (Szabo, 1996). Second, sGC is a heterodimeric protein which contains a prosthetic haem group required for stimulation by NO. It is well known that under conditions of oxidative stress, the redox state of this haem group shifts from a reduced (Fe^{2+}) state to a NO-insensitive (Fe^{3+}) state, impairing the NO-dependent activation of sGC (Stasch *et al.*, 2006; Meurer *et al.*, 2009).

To avoid the problems associated to reduced NO bioavailability, formation of ONOO^- , a promising new class of drugs has recently been developed which are NO-independent stimulators and activators of sGC. These sGC stimulators (YC-1, riociguat, BAY 41-2272) bind to the haem moiety in its normal Fe^{2+} state and increase the GTP catalytic activity independently of NO while sGC activators (cinaciguat, ataciguat, BAY 60-2770) activate the oxidized form of sGC in a haem-independent manner (Follmann *et al.*, 2013). In the context of myocardial reperfusion injury, where the oxidized sGC is expected to be the prominent form in cardiomyocytes, sGC activators are, in principle, the most interesting compounds. Administration of the sGC activator cinaciguat during reperfusion improved ventricular function in dog hearts *ex vivo* (Korkmaz *et al.*, 2009) and decreased infarct size after regional ischaemia in mice (Salloum *et al.*, 2012), rats (Krieg *et al.*, 2009) and rabbits (Krieg *et al.*, 2009; Cohen *et al.*, 2010). More recently, administration of ataciguat during reperfusion reduced infarct size in an *ex vivo* and *in vivo* rat model in a concentration-dependent manner (Inserte *et al.*, 2014). In this study, protection was reported at moderate concentrations that were effective in reducing phosphorylation of PLB at Ser^{16} and Ca^{2+} oscillations and in favouring Ca^{2+} normalization in isolated cardiomyocytes, while failing to confer protection at high concentrations lacking effect on PLB. In a recent study, it has been demonstrated that either the sGC stimulator BAY 41-2272 or the activator BAY 60-2770 perfused during early reperfusion limit infarct size in an *ex vivo* rat model, suggesting that both reduced and oxidized pools of sGC are simultaneously present during reperfusion (Bice *et al.*, 2014). Interestingly, the co-perfusion of both drugs resulted in high cGMP increase and greater infarct size compared with the sGC activator alone, supporting the idea that an excess of cGMP is detrimental.

NP/pGC pathway

There is solid experimental evidence supporting the use of NPs to stimulate pGC as a therapeutic strategy for protecting the myocardium against AMI. Stimulation of pGC by atrial natriuretic peptide (ANP) and brain natriuretic peptide administered at reperfusion has consistently shown to reduce myocardial infarction in isolated cardiomyocytes, *ex vivo* and *in vivo* hearts in a concentration-dependent manner (Philipp *et al.*, 2006; Burley and Baxter, 2007; Ren *et al.*, 2007; Gorbe *et al.*, 2010). Our laboratory reported that administration of the NP urodilatin during the first minutes of reperfusion increases cGMP levels, attenuates hypercontracture and reduces infarct size in isolated hearts (Inserte *et al.*, 2000). Furthermore, i.v. doses of urodilatin at the time of reperfusion in the *in situ* pig model subjected to transient coronary occlusion resulted in a rapid normalization of myocardial cGMP concentration in the absence of significant haemodynamic effects and attenuated infarct size. Interestingly, urodilatin at

doses resulting in excessive increase in myocardial cGMP was less cardioprotective (Padilla *et al.*, 2001).

Inhibition of cGMP degradation

Although early research has mostly focused on the stimulation of the cGMP synthesis, it has been more recently demonstrated that cGMP degradation by PDEs plays a critical role in the control of cGMP effects. Preservation of cGMP levels by pharmacological blockade of PDE5 has demonstrated to limit infarct size in different experimental models and species when PDE5 inhibitors are administered prior to ischaemia (Kukreja *et al.*, 2011). The mechanisms proposed involve PKG-dependent phosphorylation of ERK and GSK3 β (Das *et al.*, 2008), increased NO generation through activation of eNOS/inducible NOS, activation of PKC and opening of mito-KATP channels (Kukreja *et al.*, 2005). However, the evidence supporting PDE5 inhibition at the time of reperfusion as a cardioprotective strategy is less consistent. Continuous infusion or bolus injection of sildenafil and vardenafil at reperfusion reduced infarct size in *in vivo* rabbits and rats (Salloum *et al.*, 2007; Ebner *et al.*, 2013), in contrast to the findings of Reffelmann and Kloner who failed to demonstrate a cardioprotective effect with sildenafil in the same model using similar doses (Reffelmann and Kloner, 2003). It is important to note that sildenafil at concentrations that has been reported to limit infarct size did not elevate total cGMP beyond control level (Elrod *et al.*, 2007; Madhani *et al.*, 2010; Ebner *et al.*, 2013). This lack of increase in cGMP observed with sildenafil despite its cardioprotective effects has been explained by the heterogeneous compartmentalization of cGMP within the cell (Castro *et al.*, 2006) and suggests that local elevation of cGMP and no elevation in total cGMP content might be sufficient for protecting the heart. However, as total cGMP levels are severely depleted during prolonged ischaemia, it is possible that PDE inhibition at the onset of reperfusion may not effectively increase cGMP if cGMP synthesis remains depressed because of the oxidative stress and other reasons.

Pharmacological stimulation of the cGMP/PKG pathway in clinical trials

Although the cGMP/PKG pathway to date is one of the pharmacological targets for limitation of reperfusion injury with more solid preclinical evidence, only two clinical studies have directly assessed the effectiveness of this strategy in patients with STEMI using different approaches: stimulation of pGC with ANP and of sGC with nitrites respectively.

Consistent with the solid preclinical data previously described, in the J-WIND-ANP trial, stimulation of the pGC with continuous i.v. infusion of ANP during 3 days following reperfusion by either PCI or thrombolytic therapy reduced enzymic release and improved ventricular function at 6 to 12 months compared with controls (Kitakaze *et al.*, 2007).

Recently, the final results of a multicentre trial investigating the effects of i.v. sodium nitrite administered immediately prior to PCI in 118 patients with first STEMI (NIAMI) have been published (Siddiqi *et al.*, 2014). The study showed no significant differences between patients receiving nitrite or

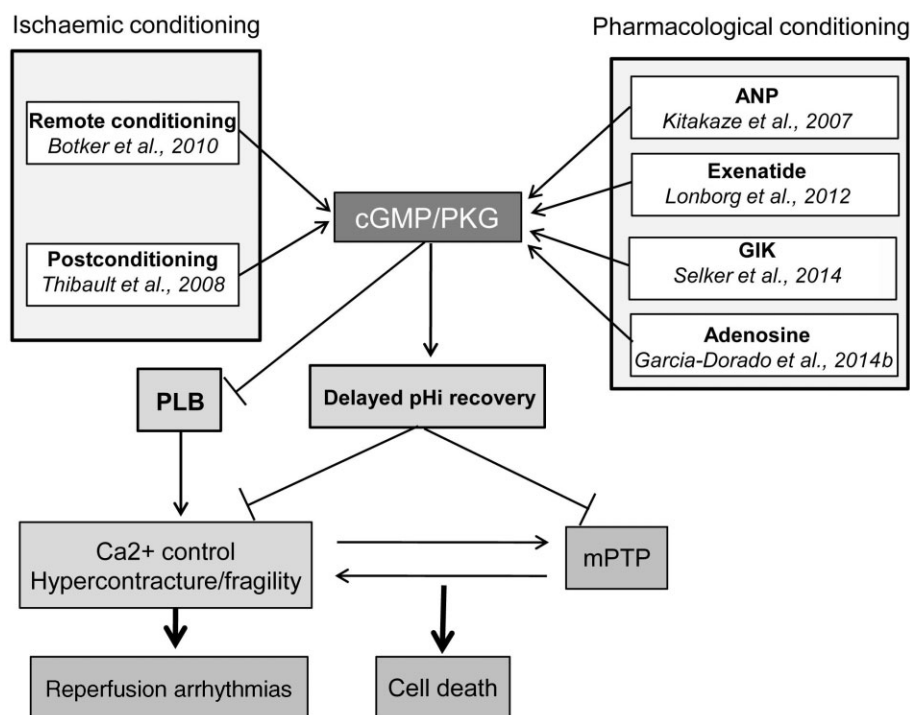


Figure 3

Proposed mechanisms of action of therapeutic interventions that stimulate the cGMP/PKG pathway and have been shown to limit infarct size in clinical trials.

placebo neither in the primary end point (infarct size at 6–8 days measured by NMR) nor in the secondary end points (acute enzyme release and ventricular function at 6 months). The failure of nitrites to protect was foreseeable from the contradictory preclinical evidence supporting a protective effect of NO donors when administered at reperfusion in animal models, as it has been discussed earlier, and reinforce the premise that pharmacological therapies should be selected on basis of a robust cardioprotection in different experimental models before being translated to the clinical setting (Hausenloy *et al.*, 2013). The ongoing NITRITE-AMI trial, in the same clinical context of patients with STEMI, analyses whether an intracoronary injection of nitrite, initiated prior to establishment of full reperfusion, reduces infarct size (NITRITE-AMI: ClinicalTrials.gov: NCT01388504).

In addition to these studies, there are a number of small clinical trials using emerging pharmacological treatments for preventing lethal reperfusion injury that, although not specifically addressed at the cGMP/PKG pathway, mediate their effects at least in part through stimulation of this pathway (Figure 3). In general, preclinical studies demonstrate that these therapies have in common the use of agonists for G_i-coupled receptors that stimulate the eNOS/sGC/PKG axis via activation of PI3K/Akt. This is the case of insulin which has been shown to be protective at the onset of reperfusion in different experimental models and is the main component of the glucose-insulin-potassium (GIK) therapy. It has been shown that insulin protects cardiomyocytes against reoxygenation injury by accelerating cytosolic Ca²⁺ recovery through a mechanism that involves increased eNOS and PKG

activities (Abdallah *et al.*, 2006). Although the first attempts to translate the administration of GIK into the clinical practice gave negative results probably because of the late administration of the treatment (Diaz *et al.*, 1998; Ceremuzynski *et al.*, 1999; Mehta *et al.*, 2005), a recent study has assessed the effectiveness of its delivery in the ambulance and in the receiving hospital for 12 h in total to patients with suspected acute coronary syndrome. The results after 30 days and 1 year follow-up demonstrate improved clinical outcomes in those patients with STEMI (reduced composites of cardiac arrest or 1 year mortality, and of cardiac arrest, 1 year mortality or heart failure hospitalization) (Selker *et al.*, 2014).

Adenosine is another G_i-coupled receptor agonist that activates Akt and therefore the cGMP/PKG pathway (Yang *et al.*, 2010). Intravenous administration of adenosine or its synthetic agonist AMP579 in the AMISTAD and ADMIRE trials provides inconclusive results with regard to their clinical use (Mahaffey *et al.*, 1999; Kopecky *et al.*, 2003). However, a subsequent reanalysis of the AMISTAD-II gave evidence of protection in those patients receiving early reperfusion (Kloner *et al.*, 2006). More recently, the effect of adenosine delivered in the form of intracoronary injection before reperfusion on the infarct size measured by NMR has been tested in the PROMISE trial (Garcia-Dorado *et al.*, 2014b). Although adenosine failed in its primary end point when considering all patients, the results showed that adenosine limits infarct size and improves ejection fraction in those receiving PCI within the first 3 h after pain onset.

There is solid experimental evidence that stimulation of the glucagon-like peptide-1 (GLP-1) signalling pathway using

agonists of GLP-1 or inhibitors of its degradation attenuate reperfusion injury through cGMP/PKG, among other pathways (Ban *et al.*, 2008; Timmers *et al.*, 2009). Two clinical trials have evaluated the effect of the GLP-1 agonist exenatide administered intravenously in patients with STEMI 15 min prior to PCI and for 6 h, and in both studies exenatide increased myocardial salvage (Lonborg *et al.*, 2012; Woo *et al.*, 2013).

Conclusions/limitations

Extensive preclinical evidence supports a pivotal role for the cGMP/PKG pathway as a survival signal and demonstrates that its stimulation at the onset of reperfusion represent a promising therapeutic target to limit infarct size in patients with STEMI. However, although several strategies applied before experimental ischaemia have been shown to enhance cGMP levels and limit cell death, many of these interventions provide less consistent results when initiated at reperfusion mainly because of the negative effects caused by the burst of ROS generated at the onset of reperfusion. This is the case for nitrites, nitrates and synthetic NO donors, stimulators of NOS activity (L-arginine, BH4) and inhibitors of PDE5. In contrast, accumulating evidence suggests that stimulation of pGC with NPs and the new class of NO- and haem-independent sGC activators are the strategies with more potential for being translated into the clinical setting for the benefit of patients with acute coronary diseases.

A potential inherent limitation of interventions aimed to stimulate the cGMP/PKG pathway is that the cardioprotective effects of cGMP are only demonstrated within the physiological concentration range. An excessive stimulation of this pathway resulting in a too large cGMP concentration appears to be detrimental (Padilla *et al.*, 2001; D'Souza *et al.*, 2003; Bice *et al.*, 2013; Inserte *et al.*, 2014). This bell-shaped dose-response curve can be explained at least in part by the complex cross-talk between the effects of cAMP and cGMP. cGMP controls cAMP-mediated signalling in cardiac myocytes by activating or inhibiting PDEs that hydrolyze cAMP. As the binding affinity of myocardial PDEs for cGMP and cAMP is different for each isoform, the global effect is expected to vary depending on the concentration of cGMP (Zaccolo and Movsesian, 2007; Stangherlin *et al.*, 2011; Inserte *et al.*, 2014). In addition, it has been described that high concentrations of cGMP may directly activate PKA and counteract the effects of PKG (Worner *et al.*, 2007).

More proof-of-concept clinical studies with infarct size as main end point are necessary to demonstrate the effectiveness and safety of pharmacological stimulation of the cGMP/PKG pathway during myocardial reperfusion, to determine which of the available drugs are more adequate, to know what is the effect of age and co-morbidities on their efficacy and safety and to establish the potential for combination with other cardioprotective treatments as RIC. However, the definite proof of the utility of these treatments necessary for their inclusion in routine clinical practice will be established only after completion of adequately designed and sized clinical trials with clinical primary end points including mortality and incidence of heart failure.

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Conflict of interest

None declared.

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