Myocardial protection against reperfusion injury: The cGMP pathway

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Summary
Reperfusion injury may cause myocardial cell death and limit the benefit achieved by restoration of coronary artery patency in patients with acute myocardial infarction. The mechanism includes altered Ca\textsuperscript{2+} handling with cytosolic and mitochondrial Ca\textsuperscript{2+} overload, Ca\textsuperscript{2+}- and ATP-dependent hypercontraction, cytoskeletal fragility, mitochondrial permeability transition and gap junction-mediated propagation of cell death, as well as alterations in non-cardiomyocyte cells, in particular platelets and endothelial cells. cGMP modulates favorably all these mechanisms, mainly through PKG-mediated actions, but cGMP synthesis is altered in reperfused cardiomyocytes and endothelial cells by mechanisms that are only partially understood. Stimulation of cGMP synthesis during initial reperfusion by means of natriuretic peptides has been found protective in different animal models and in patients. Moreover, increasing evidence indicates that cGMP is an important step in signal transduction of endogenous cardioprotection. Thus, the cGMP pathway appears as a key element in the pathophysiology of myocardial ischaemia-reperfusion and as a promising therapeutic target in patients with acute myocardial infarction.

Keywords
Ischaemic heart disease, platelet physiology, acute myocardial infarction

Introduction
The effect of ischaemic heart disease on survival and quality of life of patients, and its social impact are mainly due to its ability to cause myocardial necrosis in the context of acute coronary syndrome. The extent of cardiomyocyte cell death determines left ventricular dysfunction and remodelling, heart failure, and risk of lethal arrhythmias. It is well known that the extent of cell death caused by acute coronary occlusion depends, apart from the extent of the ischaemic area, or area at risk, on the severity and duration of ischaemia. In fact, one of the most important advancements in the treatment of patients with acute myocardial infarction, and in cardiology in general, has been the widespread use of emergent coronary recanalisation by either thrombolytic drugs or percutaneous coronary interventions (PCI). However, it is only recently that it has been established that part of cell death caused by transient coronary occlusion occurs at the time of reperfusion. This opens the possibility of improving the effectiveness of thrombolytic therapy and PCI to spare myocardium at risk by means of coadjuvant cardioprotective treatments.

The development of these treatments is based on a proper understanding of the mechanisms of cardiomyocyte death during reperfusion. Although many aspects of these mechanisms remain to be elucidated, the last two decades have witnessed major advances in their understanding. This progress has allowed the use of different experimental strategies to decrease reperfusion injury (inhibitors of contractile activation [1], Na\textsuperscript{+}/H\textsuperscript{+} and Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers [2, 3], etc.). However, clinical applicability of these potential therapies has been scarce, in general because of the absence of specific drugs adequate for human use. In this sense, modulation of cGMP has appeared as a promising therapy since several pharmacological agents have been described that can be used in humans without significant secondary effects (4, 5).

I. Myocardial necrosis during the initial minutes of reperfusion
When reperfusion occurs early enough as to save myocardium that would have suffered infarction in the absence of reperfusion,
myocardial cell death occurs mainly as necrosis, with rupture of cell membranes and release of intracellular contents, during the initial minutes of re-flow. Histological analysis of reperfused infarcts shows that they are mainly composed of areas of contraction band necrosis (1) in which cardiomyocytes show gross and characteristic disruption of their architecture and disorganisation of sarcomeres (contraction bands). Ultrastructure shows sarcolemmal rupture and massive calcium deposits in the mitochondrial matrix and sarcolemmal rupture.

Studies in isolated cardiomyocytes have shown that reoxygenation after simulated ischaemia may result in abrupt and extreme cell shortening within minutes of reenergisation, with ultrastructural changes typical of contraction band necrosis, usually described as hypercontraction, or round-up (6). The probability of occurrence of this phenomenon upon reperfusion increases with the duration of ischaemia, and in particular with the time lapse between the development of ischaemic contraction (a marker of severe ATP depletion) and the moment of reenergisation (7). Sonomicrometric analysis of myocardial segment length allows to detect myocardial shrinkage, a reduction of diastolic length of reperfused segments below the basal value which magnitude correlates with the extent of contraction band necrosis (8). This, and a paralleled response to a vast array of maneuvers, indicates that contraction band necrosis in reperfused infarcts is the correlate to hypercontraction observed in cardiomyocytes (7).

Studies in isolated cardiomyocytes have shown that hypercontraction is caused by the coincidence of reenergisation in the presence of altered Ca\(^{2+}\) handling. During prolonged ischaemia, important changes in cytosolic composition take place (7), including intracellular and extracellular acidosis, energy depletion, Na\(^{+}\) and Ca\(^{2+}\) overload and hyperosmolality. Upon reperfusion, additional changes occur, of which Na\(^{+}\) influx associated to normalization of intracellular pH (mainly through sarcoplasmic reticulum (SR) through the SR Ca\(^{2+}\)-ATPase leading role as it may result in Na\(^{+}\)/Ca\(^{2+}\) exchange (9). At the same time, restoration of respiration allows mitochondrial re-polarisation and ATP synthesis. This favors Ca\(^{2+}\) uptake by mitochondria (through the mitochondrial Ca\(^{2+}\) uniporter) and by sarcoplasmic reticulum (SR) through the SR Ca\(^{2+}\)-ATPase (SERCA), and as a result, cytosolic Ca\(^{2+}\) levels fall rapidly even in the presence of increased Ca\(^{2+}\) influx. Accumulation of Ca\(^{2+}\) in the SR eventually tends to cause Ca\(^{2+}\) release through ryanodine receptors (RyR) and subsequent reuptake, resulting in Ca\(^{2+}\) oscillations in the presence of ATP may result in excessive contractile activation resulting in hypercontraction. Hypercontraction implies marked disorganization and collapse of the cytoskeleton, and it is favored by changes in structural proteins that alter their physical properties. Hypercontraction of isolated cardiomyocytes does not cause sarcolemmal rupture unless mechanical resistance of the cell membrane is reduced (12). The changes responsible for sarcolemmal fragility have not been completely elucidated, but calpain-dependent proteolysis of the subsarcolemmal cytoskeleton, and in particular of \(\alpha\)-fodrin, plays an important role (13, 14). A simplified scheme of the different pathological events leading to cell death during ischaemia-perfusion is shown in Figure 1.

Calpains are Ca\(^{2+}\)-dependent proteases activated by Ca\(^{2+}\) and inhibited by acidosis that play a critical role in cardiomyocyte cell death during reperfusion, when both factors – Ca\(^{2+}\) overload and normalisation of pH – concur. Calpain-mediated degradation of ankyrin, the protein that anchors the Na\(^{+}\)/K\(^{+}\) ATPase to the subsarcolemmal cytoskeleton, is an important cause for Na\(^{+}\) pump dysfunction during initial reperfusion (15). This is probably because a key event in cardiomyocyte cell death during reperfusion, since it favors Na\(^{+}\) overload, reverse-mode Na\(^{+}\)/Ca\(^{2+}\) exchange and Ca\(^{2+}\) overload, that in turns favors calpain activation in a vicious cycle. Although hypercontraction per se does not cause sarcolemmal rupture in isolated myocytes, it causes them in intact myocardium. This different behavior may be due to greater physical stress imposed by hypercontraction on sarcolemma in physically attached cells to adjacent cells and to intercellular matrix, and by osmotic cell swelling caused by rapid normalisation of extracellular osmolality (16). Sarcolemmal rupture induces massive Na\(^{+}\) influx and passage of Na\(^{+}\) to adjacent cells via gap junctions, with subsequent activation of reverse Na\(^{+}\)/Ca\(^{2+}\) exchange, which results in further Ca\(^{2+}\) overload and hypercontraction of the adjacent cell (17). Cell-to-cell propagation of hypercontraction contributes to the final extent of cell death during reperfusion, and its inhibition during the initial minutes of reperfusion reduces infarct size (18, 19).

During recent years, solid evidence has been accumulated indicating that mitochondrial permeability transition (MPT) plays a key role in cardiomyocyte necrosis during reperfusion. MPT has been documented to occur during reperfusion due to reactive oxygen species (ROS) and Ca\(^{2+}\) accumulation and to correction of intracellular acidosis, a potent inhibitor of MPT (20–22). Although it is clear that MPT can cause cytochrome C release and apoptosis, the mechanism by which it causes necrosis in reperfused myocardium is not clear. A generally accepted mechanism is that MPT causes de-energisation due to mitochondrial depolarisation and loss of ATP synthesis (23). The role of MPT in reperfusion injury has been discussed in detail elsewhere (24). However, de-energisation secondary to MPT is difficult to reconcile with the occurrence of hypercontraction: firstly hypercontraction can be prevented with a contractile blocker without effect on MPT (BDM), and secondly, the mechanism causing sarcolemmal rupture and cell death within minutes of reperfusion through MTP-induced ATP depletion is not clear. It has been proposed that MPT could impair cytosolic Ca\(^{2+}\) handling in a few mitochondria, thus favoring Ca\(^{2+}\)-dependent hypercontraction fueled by ATP synthesised by the rest of mitochondria (25).

Finally, although most of the features of necrosis secondary to transient ischaemia may be reproduced in isolated cardiomyocyte preparations and crystalloid perfused hearts, it is clear that non-cardiomyocyte myocardial cells and blood born cells contribute to reperfusion injury and cell death after transient myocardial ischaemia. Among these cells, platelets appear to play a prominent role. Platelet aggregation and dislodgment of platelets aggregates at the culprit coronary artery plaque, manifested as cyclic flow variations, may be accompanied of distal microembolisation contributing to microvascular failure and focal necrosis (26). This phenomenon may be prevented by anti-
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aggregants. Although distal microembolisation may be relevant in patients receiving PCI, it appears not to be the main mechanism responsible for platelet deposition in reperfused myocardium (27). Platelets activated during ischaemia-reperfusion adhere to microvascular endothelium of reperfused myocardium in an L-selectin-dependent way (28), and may release factors that contribute to altered Ca\textsuperscript{2+} handling and cell death in cardiomyocytes (29, 30). Neutrophil infiltration and complement activation have been described to contribute to final infarct size during the initial hours of reperfusion (31). Nevertheless, the importance of these mechanisms has been debated (32), and they appear to be more prominent during reperfusion following extracorporeal circulation (33).

2. Effects of cGMP
cGMP may interfere with many of the mechanisms of cardiomyocyte death described above (Fig. 1). Among them, cGMP has been described to attenuate contractility (34–36) during reperfusion by direct effects on the contractile apparatus, and by beneficial effects on Ca\textsuperscript{2+} handling. These include inhibition of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange and activation of SERCA through PKG-mediated phosphorylation of phospholamban (11). Increased SERCA activity favors Ca\textsuperscript{2+} uptake by sarcoplasmic reticulum and prevents progression of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release waves through the cell and Ca\textsuperscript{2+} oscillations responsible for arrhythmias and hypercontraction (37). Modulation by PKG of Ca\textsuperscript{2+} release from endogenous stores through IP\textsubscript{3} inositol 1,4,5-trisphosphate receptors (IP\textsubscript{3}R) (38) and of gap-junction opening (39) has been also described. This latter effect (reduction of gap-junction mediated communication) could limit cell-to-cell propagation of hypercontracture and necrosis. Although this action could contribute to the overall beneficial effect of the cGMP pathway on infarct size, this has not been experimentally demonstrated yet. In addition, cGMP has been proposed to activate specific protective cascades as that initiated by p38 MAPK (40), to open ATP-sensitive potassium channels (K\textsubscript{ATP}) (41) and to regulate MPT (42). cGMP has also beneficial effects on non-cardiomyocyte dependent mechanism of reperfusion injury, in particular, it inhibits platelet activation, promotes vasorelaxation (43), reduces endothelial expression of adhesion proteins (44, 45), and increases endothelial permeability (46–48).

3. Regulation of the cGMP pathway
cGMP can be synthesised by two different types of guanylyl cyclases: a nitric oxide (NO)-sensitive guanylyl cyclase (GC\textsubscript{NO}), generally known as cytosolic or soluble guanylyl cyclase, and particulate guanylyl cyclases, integral proteins of the plasmatic membrane (Fig. 2) (49). GC\textsubscript{NO} is constituted of two subunits, α and β, and two different isoforms of the α subunit (α1 and α2) and of the β subunit (β1 and β2) have been described. The α1β1 heterodimer is predominantly found in the cardiovascular system, while α2β2 has been mainly found in brain (50). At least, two different particulate guanylyl cyclases can be found in the cardiovascular system, GC-A and GC-B (51). ANP (atrial natriuretic peptide) and BNP (brain natriuretic peptide) stimulate cGMP synthesis through GC-A activation, while CNP (C-type natriuretic peptide) binds to GC-B. In addition, all three natriuretic peptides can bind to a third natriuretic receptor, NPRC (natriuretic peptide receptor-C or natriuretic peptide clearance receptor). After natriuretic peptide binding, NPRC, receptor that has no guanylyl cyclase activity, binds to the G protein eliciting Ca\textsuperscript{2+} entry through L-type channels and activation of endothelial...
nitric oxide synthase (eNOS) (52). Most cGMP actions are mediated by PKG, but also by direct effects on the phosphodiesterases that degrade cAMP, which causes a crosstalk between the cAMP/PKA and cGMP/PKG pathways.

cGMP signalling can be modulated by changes in NO or natriuretic peptide availability, in cGMP synthesis or degradation, and at the level of the final targets of cGMP action, particularly PKG and phosphodiesterases 2 and 3. Detailed information on NO regulation has been reviewed elsewhere (53–55). cGMP synthesis depends on many factors, being cell energy status one of the most important. First, GTP concentration (the substrate of the synthetic reaction) is in direct correlation with ATP. Second, stimulatory and inhibitory sites for ATP have been described in both A and B types of particulate guanylyl cyclases (56). Third, ATP availability may also affect the activity of these enzymes through modifications in its phosphorylation state (49). Rapid desensitization of the signal (57) and phosphorylation by different protein kinases, as PKC (58), PKA (58, 59) and PKG (60), have been described. Recently, some studies have challenged the classical concept of an exclusive cytosolic location of GCs. The isoform α1β1 has been found to be associated to the particulate fraction in cardiomyocytes, endothelial cells and platelets (61), and in these latter cells activation with ADP or collagen has been correlated with enzyme translocation to the membrane (61). Results obtained in our laboratory suggest that cGMP synthesis in heart cells is directly dependent on the activity of the enzyme fraction associated to the membrane (62). Besides the specific regulation of the different enzymes, there are at least two agents that could exert a general modulation of the cGMP pathway: Ca2+ and PKG. Both can regulate eNOS, NO-dependent and particulate guanylyl cyclases and several phosphodiesterase isoenzymes.

4. Effects of ischaemia and reperfusion on the cGMP pathway

The effects of ischaemia on the cGMP pathway are incompletely understood. Most of the studies have analyzed changes in NO production. NO synthase activity has generally been shown to be activated during ischaemia and reperfusion (63), nevertheless part of this activity results in superoxide ion synthesis instead of NO. This may cause a decrease in NO availability during ischaemia-reperfusion, particularly in the early reperfusion, when a burst of ROS takes place (64). Much less is known about the effects of ischaemia on cGMP synthesis and degradation. It has been described that myocardial cGMP increases after 10–25 minutes (min) of ischaemia in the isolated rat heart (40, 65), while other studies in the same model have found no change (66), or a reduction (67) after 30 min of hypoxia (67) or ischaemia (66). Reduced myocardial cGMP content has also been described in the in situ rat (68) and rabbit (69) heart after 30 min of transient ischaemia. We have systematically found, in different animal models, an acute decrease in the myocardial cGMP content after 40 min of simulated ischaemia (35, 36, 70). In fact, both the basal content of cGMP and the ability of myocaridal cells to respond to cGMP activators are decreased in isolated rat hearts (36). Discrepancies found between different researchers are possibly caused by the fact that cGMP does not decrease linearly with the time of ischaemia. cGMP synthesis is probably enhanced during the first minutes of ischaemia, normalised afterwards, and acutely decreased only after 20–30 min of ischaemia. Although ATP depletion could be the main determinant of reduced cGMP synthesis during the ischaemic period, this does not seems to be the case in some cell types. In cardiomyocytes, we have found no correlation between intracellular ATP content and cGMP synthesis stimulated by NO donors or GC-A agonists (71). In agreement with these results, Geisbuhler et al. (72) reported that a drop in the nucleotide triphosphate cell content (GTP) to less than 10% of the initial levels (after 60 min of hypoxia at pH 7.4) did not produce a decrease, but a substantial increase of cGMP synthesis after stimulation with a NO-donor. A non-expected factor that was shown to be critical in cell models for cGMP synthesis during the ischaemic period was intracellular pH. Guanylyl cyclase (both soluble and particulate) activity in cell homogenates shows a typical bell-shaped curve respect to pH, resulting in negligible cGMP synthesis at pH values close to 6.4 (71, 73). In fact, cGMP accumulation after stimulation with natriuretic petides (in cultures of cardiomyocytes and endothelial cells) or NO-donors (endothelial cell cultures) was abolished at an acidic extracellular pH in either normoxic and hypoxic conditions (71, 73). Moreover, in these cultures recovery during simulated reperfusion was closely related with intracellular pH (71, 73).

5. Myocardial protection by pharmacological modification of the cGMP pathway: a promising translation

The initial studies aimed to explore the potential cardioprotective effects of cGMP during reperfusion were carried out in iso-
lated cardiomyocytes (74). These studies demonstrated that stimulation of cGMP synthesis (Fig. 3A) or exposure to soluble cGMP mimetics prevented hypercontraction and cell death during reperfusion in cardiomyocytes exposed to simulated ischaemia (hypoxia at pH 6.4). Subsequent studies proved that L-arginine protected isolated rat hearts against reperfusion injury, and that this effect was abolished by ODQ, an inhibitor of guanylyl cyclases (35). L-Arginine was also found protective in intact pig hearts submitted to transient coronary occlusion and reperfusion (75). However, increased NO availability produced by L-argi-

**Figure 3:** Translation of stimulation of cGMP synthesis with natriuretic peptides during reperfusion from myocytes to patients. A) Demonstration in cardiomyocytes submitted to simulated ischaemia of the protective effect of administrating atrial natriuretic peptide (ANP, closed circles; compared with control cells, open circles) during the first 30 min of reperfusion as demonstrated by the prevention of hypercontracture (additional reduction in cell length during reperfusion) (modified from [34]). B) Left: normalisation of myocardial cGMP with urodilatin applied at the time of reperfusion in isolated rat hearts (modified from [36]). Middle: Plasma concentration of urodilatin (an analogue of ANP synthesised by the kidney, URO) after intravenous administration at the time of reperfusion in pigs submitted to transient coronary occlusion. The dashed area indicates the end of the ischaemic period (modified from [70]). Left: Infarct size reduction by intravenous administration of URO at the time of reperfusion in pigs (modified from [70]). C) Reduced creatin kinase (CK) release and improved left ventricular ejection fraction (LVEF) in patients from the J-Wind study with acute myocardial infarction receiving intravenous ANP at the time of reperfusion (modified from [81]).
nine administration is expected to have many effects other than increased cGMP synthesis, and requires to be administered prior to reperfusion to be protective both in isolated rat hearts and in situ pig heart.

Stimulation of particulated guanylyl cyclases by the natriuretic peptide urodilatin applied at the time of reperfusion was confirmed to increase myocardial cGMP during the initial minutes of reperfusion, attenuate hypercontraction, and reduce infarct size in isolated rat hearts (36). These studies were translated to intact pigs submitted to transient coronary occlusion receiving different intravenous doses of urodilatin during the initial minutes of reperfusion (70). This approach resulted in a rapid increase in the plasmatic concentration of the drug, and in myocardial cGMP concentration (Fig. 3B) in the absence of significant haemodynamic effects. Interestingly, in agreement with the results obtained in isolated hearts, those doses of urodilatin, that normalised cGMP concentration in reperfused myocardium, were more protective than higher doses resulting in supra-normal myocardial cGMP content in reperfused myocardium. Other laboratories have also found a consistent protective effect of interventions aimed to increase cGMP in reperfused myocardium (76–79).

The concept of cardioprotection by cGMP stimulation has been tested in patients. Intravenous atrial natriuretic peptide at the time of reperfusion in patients with acute myocardial infarction resulted in less left ventricular (LV) remodelling, as indicated by smaller end-systolic and end-diastolic volumes after one month of follow-up, as compared to controls (80). More recently, the protective effect of recombinant human ANP has been demonstrated in a double-blinded trial (81). In this study (J-wind study) 569 patients were randomly allocated to receive ANP or placebo at the time of primary PCI. Patients receiving ANP showed smaller infarct size, estimated by the curves of CK release, and higher left ventricular ejection fraction (LVEF) (Fig. 3C).

6. cGMP in pre and post conditioning

The potential involvement of cGMP in pre and postconditioning has received great attention during the last years. Although this aspect is not the focus of the present review, it deserves a comment. It has been proposed that cGMP, through the activation of PKG, is implicated in the transfer of signals triggering protection from the cytosol to mitochondria (82). G-coupled receptors triggering preconditioning activate phosphatidylinositol 3-kinase (PI3-kinase) that causes the activation of eNOS. Epidermal growth factor receptor & Akt/PKB could act as intermediate steps in this process (83). NO activates cGMP synthesis and PKG-dependent phosphorylation. PKG has been proposed to evoke opening of ATP-sensitive potassium channels (KATP) in the mitochondrial inner membrane, evoking K+ entry into mitochondria and generation of ROS. It is currently unknown how the signal goes from the cytosol to the inner mitochondrial membrane, but an intermediate step implicating the ε isoform of PKC has been suggested (84–86).

It has been proposed that preconditioning and postconditioning could share signal transduction pathways. Hausenloy et al. (87) found that activation of PI3-kinase and p42/p44 MAP kinase (ERK) during the first minutes of reperfusion is required for postconditioning protection. As discussed above, cGMP is part of the PI3-kinase pathway. These pro-survival kinases (the RISK pathway) are thought to inhibit MPT by phosphorylating glycogen synthase kinase-3β (GSK-3β) (88), but it has been suggested that attenuation of Ca²⁺ oscillations by PKG-dependent phosphorylation of phospholamban may play an important role (3) (Fig. 4). However, the existence of a common signal pathway for pre- and postconditioning is subjected to debate. For example, Cx43, a gap junction protein also present at the inner mitochondrial membrane, has been found to play a role in preconditioning (89, 90), since genetic reduction of Cx43 expression (91) or inhibition of Cx43 transport to the mitochondria (92) blunts preconditioning protection. However, Cx43 appears not to be involved in postconditioning protection (92). Moreover, delayed recovery of intracellular acidosis, able to directly inhibit hypercontraction and MPT during reperfusion (93) has been proposed to explain postconditioning protection (94). Whether cGMP is involved in the role of Cx43 in ischaemic preconditioning or in the protective effect of delayed pH recovery in postconditioning is not known.

Conclusion

The cGMP signalling pathway appears to be a key element in the pathophysiology of myocardial ischaemia-reperfusion, and a valuable target for prevention of reperfusion injury. However, an important effort is still needed to elucidate the molecular mechanisms responsible for the cardioprotective effects of cGMP during reperfusion and, at the other end of the translation path, to determine the real value of treatments aimed to increase its concentration in patients undergoing myocardial reperfusion.
References


