Novel approaches and opportunities for cardioprotective signalling through cGMP manipulation

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Enhancing the tolerance of myocardium to the lethal consequences of ischemia and reperfusion, especially myocardial necrosis (infarction) remains a highly desirable therapeutic goal. While recent advances in primary prevention and timely reperfusion have reduced early mortality from acute myocardial infarction (AMI), long term prognosis is directly related to the extent of tissue necrosis sustained during the index coronary occlusion/reperfusion insult. During the last three decades or so, sustained exploration of the endogenous physiological signalling cascades recruited to enhance tolerance, has demonstrated that there are many components to the multifaceted conditioning paradigms (pre conditioning and post conditioning). One such component is guanosine-3', 5'-cyclic monophosphate (cyclic guanosine monophosphate, cGMP), a cyclic nucleotide responsible for eliciting many potentially cardioprotective actions.

1. Historical background

cGMP is an intracellular second messenger produced from the purine nucleotide guanosine triphosphate (GTP) in a reaction catalysed by cytosolic soluble guanylyl cyclase (sGC) or membrane associated particulate guanylyl cyclase (pGC). Following the discovery of cAMP in the late 1950s\(^1\), cGMP, was confirmed to be a physiological mediator when Price et al. isolated it from rat urine in 1963\(^2\) and Sutherland’s laboratory demonstrated that steroid, thyroid and pituitary hormones affected urinary excretion of cGMP. Fifty years after its discovery, cGMP is now established as a ubiquitous second messenger, mediating a large variety of physiological processes.
Within the cardiovascular system, cGMP is well-established as an important pharmacological target. Indeed, targeting cGMP signalling for the therapeutic treatment of cardiovascular disease is not a recent discovery. T Lauder Brunton’s description of the effects of amyl nitrite in angina pectoris in 1867 and William Murrell’s subsequent description of the effects of glyceryl trinitrate (nitroglycerin) in 1879 preceded any understanding of the mechanism of action by more than a century. Following the description of a (then) unidentified endothelium-derived relaxing factor (EDRF) in the early 1980’s it was demonstrated that EDRF increases cGMP synthesis in isolated blood vessels. The subsequent identification of EDRF as nitric oxide (NO) and its association with guanylyl cyclase activity and cGMP synthesis opened up an area of biology with huge potential for pharmacological exploitation, especially in the cardiovascular system. In the early 1990’s cGMP was shown to be an important cyclic nucleotide in affording the preconditioned state in rabbit myocardium.

In the late 1980s and early 1990s, the sequential discovery of a series of peptide mediators produced within the cardiovascular system, called natriuretic peptides, opened up a new dimension in cGMP signalling. The major members of this family - in order of discovery; atrial natriuretic peptide (ANP), brain- or B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) – all elicit their biological actions, at least in part, through elevation of intracellular cGMP. The natriuretic peptide membrane-associated receptors, NPR-A and NPR-B, contain guanylyl cyclase domains capable of catalysing the conversion of GTP to cGMP. This particulate guanylyl cyclase (pGC) activity is, as far as we know, uniquely activated by the natriuretic peptide family of mediators which include ANP, BNP, CNP and the related
guanylins and atriopeptins. This further dimension to cGMP regulation in the cardiovascular system provides additional complexity and opportunities for therapeutic manipulation.

Over the last thirty years extensive investigation has focused on the physiological roles and regulation of the cGMP pathway, its disruption in various diseases and its therapeutic “druggability”. In this review we explore the extensive literature focusing on myocardial cytoprotection against ischaemia-reperfusion injury. We consider the basic research and consequent clinical studies that have followed with particular reference to the clinical treatment of acute myocardial infarction (AMI).

**2. Production and localisation of cGMP**

cGMP production is mediated by two distinct guanylyl cyclases, which are spatially discrete within the cell. pGC is described as membrane associated and is confined to the plasma membrane, connected to the extracellular ligand binding domain via a kinase homology region. ANP shows the highest affinity for the NPR-A receptor, whilst BNP is 10-fold less potent.\textsuperscript{10} CNP is the primary ligand for NPR-B, stimulating guanylyl cyclase with 50-500-fold greater affinity than ANP and BNP (Figure 1).\textsuperscript{11} sGC is found in the cytoplasmic domain, catalysing the production of cGMP primarily through the binding of the endogenous ligand NO. sGC is a heterodimeric haem protein consisting of both an alpha and beta subunit. To date, two alpha and two beta isoforms have been identified.\textsuperscript{12,13} The α1β1 protein has been most extensively researched and is found in most tissues, including the kidney, brain, heart and
vascular tissue.\textsuperscript{14} A heterodimer consisting of both subunits is essential to form the catalytically active sGC.\textsuperscript{15,16} The N-terminal histidine 105 haem binding site of the $\beta$ subunit forms the basis of the NO-sensing region, which when occupied by NO leads to a 200-fold increase in the synthesis of cGMP (Figure 1).\textsuperscript{17} Endogenous CO has also been shown to activate sGC, but producing only a 4-fold increase in activity.\textsuperscript{18}

cGMP activity is governed by specific cGMP-binding motifs on target proteins and its highly regulated degradation by phosphodiesterease (PDE’s). cGMP-dependent protein kinase (PKG) represents the primary cGMP mediator which phosphorylates target proteins. PKG-I shares its allosteric cGMP-binding motif with PKA (50-fold lower selectivity than cAMP) and a second cGMP effector, the cyclic nucleotide-gated (CNG) ion channel. The second binding motif occurs only in the cGMP-regulated PDE’s. Indeed, it is this complex combination of effectors that tightly regulates cGMP signalling. PKG, PKA and CNG channels are co-localised with effector proteins in different cell types resulting in differing responses to elevated cGMP levels. For example PKG is co localised with phospholamban (PLB) and the $IP_3$ receptor in the sarcoplasmic reticulum in smooth muscle cells. In contrast PKA is co localised with PLB and $IP_3$ in cardiac myocytes eliciting differing responses to elevation of cGMP. Disruption of cGMP signalling has been identified in many aspects of cardiovascular function and disease including platelet aggregation, inotropy, cell growth/proliferation and apoptosis, all of which rely on tightly regulated and specific signalling.

Hydrolysis of cGMP to 5’GMP occurs through PDE catalytic activity. There are ten families of mammalian PDE, four of which hydrolyse both cGMP and cAMP (1, 2, 3 and 10) and three which are specific for cGMP (5, 6 and 9). Specific targeting sequences found in PDE’s traffic them to specific proteins leading to their being
expressed in discrete subcellular locations. The net effect of this is that the intracellular concentration of cGMP varies within different subcellular compartments. Of the PDE isozymes, PDE 2 and 5 are of particular interest because they have been demonstrated to show distinct regulation of specific cGMP subcellular concentrations as a result of pGC and sGC mediated production respectively.\textsuperscript{19} Thus, there is a multifaceted approach to regulating cGMP and its downstream effectors. In this review, we will focus on potential therapeutic targets that can augment the production, distribution, localisation and metabolism of cGMP to limit the cytotoxic affects of myocardial ischaemia-reperfusion injury, resulting in myocardial necrosis.

3. NO, Nitrite and Nitrate

\textit{Nitric oxide}

NO is a gaseous autacoid formed by the action of nitric oxide synthase (NOS) on L-arginine, NADPH and molecular oxygen.\textsuperscript{20} NO is a critical regulator of cardiovascular homeostasis.\textsuperscript{20} Under physiological conditions, NOS mediated NO formation is permitted due to uncompromised delivery of substrate and molecular oxygen. However in disease states such as coronary artery disease characterized by abnormally low oxygen and high energy phosphate depletion, endothelial derived NO bioavailability as a result is severely compromised.\textsuperscript{21} Acute and chronic hypoxia down-regulates endothelial NOS (eNOS) at mRNA and protein levels in various cardiovascular tissues.\textsuperscript{22} For a comprehensive review of NO in ischaemic heart disease we direct the reader to Rassaf \textit{et al.}\textsuperscript{23} and Ferdinandy and Schulz\textsuperscript{24}. 
Nitrate and nitrite as a potential NO reserve

An alternative pathway for NO formation involving nitrate and nitrite, which predominates when tissue oxygen tension is low, has been described in mammals.\textsuperscript{25} Nitrate ($\text{NO}_3^-$) and nitrite ($\text{NO}_2^-$) can be considered reservoirs for NO when NOS-dependent NO levels are insufficient to maintain cellular homeostasis.\textsuperscript{21} $\text{NO}_3^-$ and $\text{NO}_2^-$ can be reduced in sequential steps by enzymatic and non-enzymatic mechanisms creating new pools of NO with the potential to activate the sGC/cGMP/PKG pathway or by other processes that are distinct from this classical route of intracellular signalling.\textsuperscript{21} The reaction of NO with oxyhaemoglobin leads to the production of nitrate and the formation of methaemoglobin.\textsuperscript{26,27} Green leafy vegetables are a major source of NO capable of delivering more nitrate than all the NOS isoforms combined.\textsuperscript{21,28} $\text{NO}_2^-$ is formed following auto-oxidation of NO, non-enzymatically by deoxyhaemoglobin,\textsuperscript{29} and by the enzymes ceruloplasmin, xanthine oxidoreductase and aldehyde oxidase.\textsuperscript{30} The mitochondrion has been shown to be a source of $\text{NO}_2^-$, and to exhibit nitrite reductase activity.\textsuperscript{31} Plasma nitrate concentration in humans is approximately two orders of magnitude greater than that of nitrite ranging from 20-50 μM\textsuperscript{32}, plasma nitrite concentrations in fasting humans are typically within the range 50-500 nM.\textsuperscript{33-36}

Both $\text{NO}_3^-$ and $\text{NO}_2^-$ were considered terminal products of NO synthesis, but are now recognized as reserve sources for NO production, especially under conditions of hypoxia.\textsuperscript{31} NO derived particularly from nitrite has been shown to modify proteins and redox state,\textsuperscript{37} which may be determinant factors in facilitating cytoprotection in models of ischaemia-reperfusion injury in the heart and other organs.\textsuperscript{38} The precise
mechanism by which NO\(_2^-\) is reduced to NO or whether NO is released from erythrocytes to promote vascular smooth muscle relaxation is currently unclear.\(^{31}\) Nitrite-derived NO\(^{39}\) and dinitrogen trioxide (N\(_2\)O\(_3\))\(^{40}\) have been proposed as nitrogen oxide species responsible for promoting NOS-independent vasodilatation in humans. Lunberg and colleagues\(^{21}\) suggest that both species form in the circulation, the former liberated by the reaction of nitrite with deoxyhaemoglobin, and the latter from the reaction of nitrite-bound methaemoglobin and NO, forming a nitrogen dioxide radical which then reacts with free NO to form N\(_2\)O\(_3\).

_Nitrate, nitrite and nitric oxide signal transduction mechanisms: cGMP vs S-nitrosylation_

The mechanisms by which nitrate and nitrite are converted to NO have already been highlighted; it is presumed that this pool of NO is capable of activating the same intracellular pathways as NOS-derived NO. NO stimulates sGC leading to the generation of cGMP, thereby affecting downstream mechanisms that modulate cell function. This classical form of NO signaling has been discussed extensively in comprehensive reviews.\(^{20,41}\) NO derived from inducible NOS (iNOS) has a pertinent role in regulating and contributing to signalling associated with oxidative stress in pathological states including inflammation. However the numerous intricate mechanisms and processes involved are beyond the scope of this review. We would refer readers to excellent reviews by Pfeilschifter et al\(^{42}\), Danson et al\(^{43}\), and Klein\(^{44}\) for a fuller examination.
There is growing interest in another form of NO signaling independent of sGC. This involves modification of protein thiol groups. S-nitrosylation implies the binding of NO to cysteine residues, altering the stability, binding, activity, localization and irreversible oxidation of cysteine rich proteins which are numerous.\textsuperscript{37,45,46} For example, s-nitrosylation increases sarcoplasmic/endoplasmic reticulum ATPase (SERCA) activity\textsuperscript{47}, increases connexin 43 gap junction communication\textsuperscript{48} and inhibits phosphatase and tensin homolog (PTEN) activity\textsuperscript{49} - preventing inhibition of PI3K/Akt, which are significant factors in the context of myocardial ischaemia-reperfusion and cardioprotection.\textsuperscript{37} Ischaemic preconditioning effected by brief periods of ischaemia and reperfusion prior to a sustained ischaemic insult is cardioprotective. Mouse hearts subjected to this form of cardioprotective intervention were found to have increased s-nitrosylation of L-type calcium channels and SERCA decreasing intracellular calcium load, and S-nitrosylation of the F\textsubscript{1}-ATPase limiting ATP breakdown.\textsuperscript{47}

\textit{The cytoprotective action of nitrite}

Organic and inorganic nitrate and nitrite therapy has many beneficial effects in the cardiovascular system. Numerous studies during the last two decades have examined the protective actions of organic nitrates and other NO-donor compounds in models of cardioprotection against ischaemia-reperfusion injury and have demonstrated the ability of the acutely administered agents to limit infarct size. Preliminary data has demonstrated that nitroglycerin can limit infarct size in isolated rat hearts when administered during early reperfusion.\textsuperscript{50} More recently however, the potential of inorganic nitrite and naturally occurring dietary nitrite as cardioprotective
sources of NO has been examined. Sodium nitrite (10-100 μM) reduced infarct size by some 70%, in the rat isolated heart. The protective effect of nitrite given for the full duration of global ischaemia but not reperfusion, was reversed by the NO-scavenger carboxy-PTIO, suggesting that nitrite mediated cardioprotection is in part NO-dependent. Nitrite cardioprotection appears to be NOS-independent as it was shown that sodium nitrite limitation of infarct size in a mouse model was still present during pharmacological NOS inhibition or in eNOS knockout mice. Other studies implicate xanthine oxidase activation and K<sub>ATP</sub> channel opening in sodium nitrite-induced infarct size limitation in the rat heart in vivo and ex vivo. There is evidence to suggest that nitrite-derived NO S-nitrosylates mitochondrion complex-1 during ischaemia-reperfusion, delaying mitochondrial permeability transition pore (mPTP) opening, ROS formation and cytochrome c release, and ATP depletion, thus conferring cardioprotection. Shiva and colleagues found that sodium nitrite given 24 hr prior to ischaemia or acutely prior to reperfusion caused a marked reduction in infarct size in mouse hearts. Furthermore, it was demonstrated that sodium nitrite improves mitochondrial respiration following hypoxia in a concentration-dependent manner and delayed mPTP opening and cytochrome c release in isolated mitochondria subjected to hypoxia and reoxygenation. In contrast to these data it has been demonstrated that NO can inhibit cytochrome c oxidase (complex IV) activity, in mitochondria isolated from rat brain and skeletal muscle following incubation with SNP and GSNO respectively.

Bryan and co-workers found that mice given free access to chow and water supplemented with sodium nitrite for 7 days were more resistant to myocardial ischaemia-reperfusion injury, compared to mice on a standard diet, displaying a 48% relative reduction in infarct size compare to mice that did not receive sodium nitrite.
Furthermore, dietary sodium nitrite significantly increased plasma and tissue RXNO products, for example S-nitrosothiols and nitrosoproteins, increased steady-state concentrations of nitrate and nitrite, and nitrosyl-haem products. These hallmark features are likely to be very important in conferring nitrite protection of ischaemic myocardium. Most recently it has been demonstrated that acute ingestion of dietary nitrate in the form of beetroot juice elevated circulating nitrate and nitrite levels in humans and elevated platelet cGMP levels in healthy male subjects only. Although this evidence is not associated with cardiovascular cytoprotection, it highlights the potential for dietary or supplemented nitrite as a credible mechanism for cGMP elevation. Previously the authors demonstrated that beetroot juice lowered blood pressure in healthy volunteers, elevating plasma NO\textsubscript{2} and cGMP levels.

Pluta and colleagues examined the safety and feasibility of long-term NO\textsubscript{2} therapy in healthy volunteers administered sodium nitrite intravenously for 48 h. Cardiac NO\textsubscript{3}, NO\textsubscript{2} and S-nitrosothiol levels in plasma increased in all subjects and returned to pre-infusion baseline levels within 12 h. The authors concluded that prolonged NO\textsubscript{2} iv. infusion below the maximal tolerated dose is safe for use in treatment of major diseases including ischaemic heart disease. Side effects were limited to asymptomatic transient decreases in arterial blood pressure and asymptomatic increases in methaemoglobin. Indeed, the Nitrites In Acute Myocardial Infarction (NIAMI) trial is a placebo-controlled, double-blind, phase-2 clinical trial that aims to determine whether a 5 min iv infusion of sodium nitrite (14 μmol/mL) just prior to PCI can reduce infarct size and associated enzyme biomarkers including creatine kinase and troponin I, in patients presenting with ST-elevation MI (STEMI). Other endpoints include left ventricular ejection fraction and end diastolic volume at one
week and six months after MI, and infarct size measured by magnetic resonance imaging at six months post MI. This multi-centre clinical study is the first investigating whether sodium nitrite infusion can reduce myocardial infarct size in man. This study may provide a significant platform for establishing sodium nitrite as first-line therapy in the clinical treatment of AMI.

*Receptor mediated NOS activation*

Many receptors have been linked to PI3K/Akt/eNOS signalling and elevation of intracellular NO including bradykinin, adenosine and opioid peptide receptors. Most recently β3-AR stimulation (see Figure 2) has been demonstrated to stimulate NO generation via eNOS in the left and right human myocardium, which is blocked in LV by the PI3K inhibitor LY294002. Aragon and colleagues recently demonstrated that β3-AR stimulation limits infarct size when administered at reperfusion in mice. This protection was accompanied by an elevation in eNOS-PSer1177 and elevated nNOS levels. Whether this protection is mediated by sGC/cGMP signalling or possible inhibition of mitochondrial respiration and xanthine oxidase remains to be elucidated.

4. NO-independent stimulation/activation of sGC

In addition to elevating cGMP via manipulation of NO, targeting sGC directly has become tractable in recent years. In this section we discuss the redox that sGC exists in, how the different redox states can be specifically targeted and the possibility of utilising CO as an alternative ligand.
Soluble guanylyl cyclase redox

The rate of reaction in physiological conditions is regulated by diffusion; however complex redox equilibrium of sGC exists, identifying challenges in successfully targeting this pathway. It is now well accepted that sGC can exist in three different forms depending on the redox state of the central haem group. The reduced (ferrous) haem group is critical for NO-sensing and stimulation of the enzyme. The oxidised (ferric) form is insensitive to NO, and appears to be physiologically unimportant in cGMP signalling. In addition a haem-free state exists, which, like the oxidised state, does not elicit enzymatic activity via NO. Identification of the three states of sGC has led to proposals that a redox shift occurs in pathological conditions. A shift in redox state from the ferrous to ferric state is associated with oxidative stress and production of ROS. This has been identified as one of several mechanisms in which the sGC/NO signalling pathway can be disrupted. Stasch et al. confirmed the presence of a sGC indistinguishable from the oxidised form of sGC found in vitro, in rat aorta, porcine pulmonary arterial endothelial cells and human platelets.

Direct cGMP targeting

Increasing intracellular concentration of cGMP has been shown to reduce myocyte contractility during reperfusion by inhibition of Na\(^+/\)Ca\(^{2+}\) exchange and activation of SERCA via PKG mediated phosphorylation of PLB. PKG has also been shown to activate BK\(_{\text{Ca}}\) channels which when inhibited at reperfusion abolish protection afforded by upstream targets of cGMP.
D’Souza et al.\textsuperscript{70} reported that low concentrations of 8-Br-cGMP, a synthetic cGMP analogue given just prior to ischaemia through to early reperfusion limited infarct size in a rat isolated heart model. It was later shown that 8-Br-cGMP also produced infarct limitation when given at reperfusion, reducing infarct size by 40\% compared to control hearts.\textsuperscript{71} These data demonstrated that targeting cGMP/PKG directly could afford infarct limitation mediated by elevating cGMP. 8-Br-cGMP has been reported to activate p38 MAPK in isolated adult rat cardiomyocytes, but not Erk1/2 suggesting further components of the cytoprotective mechanism.\textsuperscript{72}

\textit{sGC stimulating compounds}

The phenomenon of “nitrate tolerance” (tachyphylaxis to the vasodilator effects of organic NO donors) drove the discovery from the 1990s onwards of compounds that can initiate the sGC/cGMP pathway independently of NO or when bioavailability of NO is low. Ko \textit{et al.}\textsuperscript{73} described an indazole derivative, YC-1 (5-[1-(phenylmethyl)-1H-indazol-3-yl]-2-furanmethanol), later described as the first NO-independent, haem-dependent stimulator of sGC.\textsuperscript{74} Subsequently, structurally related compounds were discovered that stimulate sGC, including BAY 41-2272 (3-(4-amino-5-cyclopropylpyrimidine-2-yl)-1-(2-fluorobenzyl)-1H-pyrazolo[3,4-b]pyridine) and BAY 63-2521/Riociguat (methyl N-[4,6-diamino-2-[1-[(2-fluorophenyl)methyl]-1H-pyrazolo[3,4-b]pyridin-3-yl]-5-pyrimidinyl]-N-methyl-carbaminate)\textsuperscript{75}, (see Table 1) The mechanisms by which these compounds stimulate the activation of sGC are complex and go beyond the scope of this review.\textsuperscript{73,75,76} However their use has been investigated in pathological conditions where NO formation is compromised or when nitrate tolerance has developed.\textsuperscript{77}
sGC activating compounds

A second distinct class of sGC modulating compounds, described as sGC activators was subsequently discovered. High throughput screening revealed several aminodicarboxylic acids including BAY 58-2667 (4-(4-carboxybutyl)(2-((4-phenethylbenzol)oxy)phenethyl)amino)methyl)benzoic acid). Described as the first and most potent NO-independent, haem-independent sGC activator, Stasch et al. demonstrated that BAY 58-2667 activated sGC even after it had been oxidised by the sGC inhibitor ODQ, or even after losing its haem group completely. This class of compounds has become favoured for use in the exploration of pathological diseases as they target the oxidised (pathological) state of the enzyme. In light of the proposed shift towards the oxidised and haem-free states under conditions of oxidative stress these compounds have been utilised in experimental models of ischaemia-reperfusion where oxidant conditions are well documented. Investigation of the chemistry and pharmacology of these compounds demonstrates that sGC activation goes beyond NO binding to the haem site on the β subunit.

Specific sGC targeting

Krieg et al. demonstrated significant infarct reduction in the rat heart when the sGC activator BAY 58-2667 was given at reperfusion following 30 min global ischaemia. They reported an elevation in cGMP levels in hearts perfused with the activator 40 times greater than in control hearts and further demonstrated that infusion of BAY 58-2667 in open chest in situ rabbits following left coronary artery occlusion
limited infarct size by 40%. The protection was independent of NO, but dependent on PKG and mK$_{ATP}$ signalling. In a similar model Cohen et al.$^{80}$ reported reduction in infarct size of 54%. Interestingly the protection in that study was abrogated in the presence of a NOS inhibitor, an observation not concordant with the literature describing BAY 58-2667’s mechanism of action.$^{78}$ Most recently Salloum et al.$^{81}$ have demonstrated that BAY 58-2667 can limit infarct size when administered pre- or post-ischaemically in adult rabbits. They further suggested that PKG activity was increased following perfusion and interestingly H$_2$S levels were elevated, abrogated by the PKG inhibitor KT5823. These data corroborate the most recent study which demonstrates that BAY 58-2667 limited infarct size at reperfusion in mouse. This protective effect was abrogated when the same treatment protocol was utilised in cardiomyocyte specific PKG-I knockout mice.$^{82}$ Work from our own laboratory has demonstrated that perfusion of a structurally similar sGC activator BAY 60-2770 could afford infarct limitation when perfused during reperfusion in an isolated rat heart model of regional ischaemia.$^{83}$ This protection was not blocked by concomitant perfusion of ODQ, a haem site oxidiser. These data demonstrate that the ferric form of sGC is present in rabbit, rat and mouse myocardium during ischaemia and early reperfusion as an elevation in cGMP was measured following perfusion of the sGC activator in this setting.

*Haem oxygenase and CORMs*

The microsomal enzyme haem oxygenase (HO) catalyses the degradation of haem to CO, producing bilirubin and iron as co-products. HO-1 is not constitutively present but can be activated under many pathological stimuli, including those present during
ischaemia-reperfusion such as oxidative stress and hypoxia.\textsuperscript{84} Although CO mediated activation of sGC only elicits small increases in catalytic activity, the antioxidants NAC and ALF were able to limit infarct size in diabetic rats, which was abrogated by HO-1 inhibition.\textsuperscript{85} cGMP levels were shown to be elevated in the aorta of spontaneously hypertensive rats following administration of the same HO-1 inducing agent, hemin. Induction of HO-1 by CO releasing molecules (CORMS) has been shown to be cytoprotective when administered prior to ischaemia-reperfusion.\textsuperscript{86} The authors reported that the protection observed is comparable to that seen in classical late phase pre-conditioning; protection was conferred in part by recruiting HO-1. Taken together these studies demonstrate that HO/CO/sGC/cGMP signalling is impaired under pathological conditions in the myocardium, offering an additional target to afford cytoprotection (Figure 2).

5. Natriuretic peptide signalling

\textit{Natriuretic peptides as an indicator of infarct size}

NPs play a fundamental role in cardiovascular homeostasis, their release being primarily regulated by pressure overload and myocardial stretch. In 1991, Mukoyama \textit{et al.}\textsuperscript{87} reported for the first time that BNP levels were elevated during myocardial infarction. It was later reported that elevation of BNP correlates with ischaemia severity.\textsuperscript{88,89} Since then several experimental studies have documented elevation of ANP\textsuperscript{90-92} and BNP\textsuperscript{70,93} in models of myocardial ischaemia. Most recently, numerous clinical studies confirmed the usefulness of measuring BNP levels in patients presenting with STEMI, and suggest that they are a good indicator of prognosis and infarct size.\textsuperscript{94-96}
Natriuretic peptides at reperfusion

Several studies have identified that NPs administered at reperfusion afford protection in both rat and rabbit models of ischaemia-reperfusion. Yang et al.\textsuperscript{97} reported that administration of ANP just prior to reperfusion limited infarct size in the rabbit heart. They also demonstrated that the protection afforded required $K_{\text{ATP}}$ activity as inhibition of the channel with 5-HD abrogated the protective effects of ANP. BNP was also shown to limit infarct size in an \textit{in situ} rat heart model, limiting infarct size in a concentration dependent manner.\textsuperscript{98} Our laboratory reported similar results in an \textit{ex vivo} rat heart model, demonstrating concentration dependent infarct limitation with BNP, which like previous studies was dependent on $K_{\text{ATP}}$ channel activity\textsuperscript{69}. Furthermore, we reported that NPs could play a role in post-conditioning mediated protection as the non-specific NPR-A/NPR-B antagonist isatin abolished the infarct limitation afforded by a 6 x 10 sec post-conditioning protocol in the rat heart.\textsuperscript{69} In a cell viability assay it has been reported that along with the sGC activator SNAP, BNP can limit cell death via common downstream signalling pathway involving elevated PKG.\textsuperscript{99} Most recently, George \textit{et al.}\textsuperscript{100} reported that perfusion of BNP for 7 days post AMI significantly improved LV function and decreased LV remodelling in the rat heart. ANP was administered to patients in the J-WIND clinical trial in which infarct size measured by creatine kinase was accessed. Reported outcomes of the trial suggested that patients receiving ANP had lower infarct size, fewer reperfusion injuries and better outcomes than controls\textsuperscript{101} (Table 2).

Alternative natriuretic peptide signalling mediates infarct limitation
Infarct limitation afforded by NPs has been demonstrated to require $K_{ATP}$ activity as described above. It has also been demonstrated that protection is mediated by elevation of cGMP and distal PKG targets converging on the same effectors as NO/sGC signalling, i.e. regulating calcium via PLB and L-type calcium channels and potassium efflux through the $K_{ATP}$ channel.\textsuperscript{41,70,102}

Other signalling pathways have been suggested to play a part in NP mediated infarct limitation. D’Souza \textit{et al.}\textsuperscript{103} reported that concomitant perfusion of L-NAME prior to LDCA occlusion in the rat heart abrogated the protection afforded by BNP alone. Similarly, perfusion of ODQ abolished BNP induced infarct limitation\textsuperscript{103}. These results suggested that activation of NOS and sGC are required to afford BNP protection. Whether cGMP generated by pGC, sGC or both was needed to afford BNP protection remains unclear. However these intriguing observations raise the possibility that NP-induced cardioprotection may be mediated through mechanisms that are more complex than activation of NPR-A alone.

Similar observations were made in a reperfusion targeted treatment by Ren \textit{et al.}\textsuperscript{98}, who report that L-NAME reversed the protection afforded by BNP when given just prior to and throughout reperfusion in an \textit{in situ} rat heart model. This was supported by Western blotting analysis that demonstrated that a cardioprotective dose of BNP significantly increased eNOS expression. These results were corroborated by our laboratory when L-NAME administered at reperfusion in an \textit{ex vivo} rat heart model abrogated BNP infarct limitation.\textsuperscript{69} In an isolated perfused rabbit heart model, Yang \textit{et al.}\textsuperscript{97} demonstrated that the upstream activators of eNOS are also essential for ANP mediated protection. cGMP has been thought to elicit its cytoprotective actions through recruitment of the reperfusion injury salvage kinase (RISK) pathway. Wortmannin, an inhibitor of PI3K, and PD98059, an inhibitor of ERK, independently
blocked ANP mediated infarct limitation in the rabbit heart. Furthermore Yang et al. reported that concomitant perfusion of ODQ and ANP abrogated ANP mediated infarct limitation. Taken together these data strongly suggest that a pathway converging on sGC (PI3K/Akt/eNOS/NO/sGC) plays a critical role in NP mediated infarct limitation. However, biochemical confirmation of these observations is lacking. These studies also question whether there is a direct pGC/cGMP/PKG component to cytoprotective signalling or whether pGC mediated protection occurs indirectly via PI3K/Akt/eNOS/NO/sGC.

Particulate guanylyl cyclase independent signalling

Historically regarded as a clearance receptor, NPR-C is devoid of guanylyl cyclase coupled signalling, coupled to adenyl cyclase inhibition through the inhibitory guanine nucleotide regulatory protein (Gi). NPR-C may in fact couple to signal transduction pathways in some cell types.\textsuperscript{104,105} Interestingly, CNP signal transduction via NPR-C has been shown to be cardioprotective in the rat heart.\textsuperscript{106} Surprisingly this protection was potentiated in the presence of the NOS inhibitor L-NAME. This observation is in contrast to that observed when concomitantly perfused with BNP. It remains to be determined how NPR-C signalling mediates infarct limitation. One possibility is that it is coupled to potassium channels as NPR-C activity has been linked to $K_{ir}$ channel opening in the mesenteric vasculature.\textsuperscript{107} A second hypothetical possibility is signalling through the classical PI3K/Akt/NO/sGC/cGMP cytoprotective pathway. However, abrogation of the protection by L-NAME and no net cGMP elevation when NPR-C was targeted in rat aorta VSM cells\textsuperscript{108} suggests that a modified pathway is more likely.
Most recently a new class of so-called “designer” NPs is being developed. Cenderitide (CD-NP) is the first in this class of NPs which co-activates both NPR-A and NPR-B. It has been shown to reduce LV mass in MI-model rodents and cardiac unloading in dogs. Initial clinical trials suggest a reduction in blood pressure in stable heart failure patients as well as reduced creatine levels. It is proposed that continuous infusion of cenderitide through a subcutaneous pump will improve patient outcome and reduce the duration of hospital stay. Their cytoprotective benefit in models of myocardial ischaemia-reperfusion is yet to be explored.

**Phosphodiesterases**

*Phosphodiesterase inhibition*

Maintaining cGMP levels during ischaemic insult by pharmacological manipulation of phosphodiesterases has been extensively investigated. The cGMP specific PDE5 inhibitors have been utilised to explore this mechanism. A recent study has reported that as much as 20% of cGMP degradation in human myocytes is attributable to PDE5. Expression levels of PDE5 are increased in the failing myocardium and PDE5 inhibition has been shown to limit infarct size when administered prior to global ischaemia in a mouse model. The authors demonstrate that this protection is abolished when sildenafil was concomitantly perfused with the PKG blocker KT5823. They further concluded that the protection was associated with PKG-dependent phosphorylation of ERK, GSK3β and increased expression of the Pro survival factor Bcl-2. Early investigations demonstrated that sildenafil was cardioprotective in an *in situ* rabbit model, protection which was blocked by the K{\text{ATP}} channel blocker 5-HD and PKC inhibitor chelerythrine. This has been corroborated using another
PDE5 inhibitor vardenafil in combination with either the mitochondrial $K_{\text{ATP}}$ channel blocker 5-HD or the sarcolemmal $K_{\text{ATP}}$ channel blocker HMR1098.\textsuperscript{116} It was later demonstrated that sildenafil could induce delayed preconditioning via elevation of both iNOS and eNOS. Protection was blocked by the iNOS inhibitor 1400W.\textsuperscript{117} Tadalafil, a PDE5 inhibitor with a longer half life was also able to limit infarct size in mouse \textsuperscript{118,119} and rabbit.\textsuperscript{120} More recently, protection has been demonstrated when sildenafil is perfused during early reperfusion.\textsuperscript{121} The authors report that administration of the PDE5 inhibitor was associated with PLB Ser\textsuperscript{69} phosphorylation, also PKG-dependent.\textsuperscript{122} Interestingly mouse hearts subjected to 30 min global ischaemia followed by 10 min reperfusion with sildenafil did not elevate total cGMP beyond control level. This observation is corroborated by Elrod \textit{et al.}\textsuperscript{123} who demonstrate that infarct limitation in a mouse model was independent of eNOS/NO/cGMP signalling and particularly that low level sildenafil (0.06 mg/kg) did not elevate myocardial cGMP levels. Further mechanistic studies have demonstrated that both sildenafil and vardenafil elicit their infarct limiting properties through a $K_{\text{ATP}}$ channel mediated mechanism\textsuperscript{124} and that sildenafil improves left ventricular function in mice.\textsuperscript{125}

\textit{Regulation of specific cGMP pools}

Spatially distinct pools of cGMP are now widely accepted; however, whether these discrete pools mediate individual and distinct actions remains to be fully demonstrated. Evidence supports the notion that PDEs which are localised by binding of GAF domains to proteins regulate specific cGMP pools, both in their cellular location but also via cAMP/PKA action through PDE2/3. Castro \textit{et al.}\textsuperscript{19}
reported that PKG activity can regulate cGMP concentrations in rat cardiac myocytes differentially via pGC and sGC. PKG activated via pGC stimulates further cGMP production via positive feedback, the cGMP being localised to the sarcolemma via PDE2 control. Conversely sGC mediated PKG activity further enhances PDE5 activity limiting cGMP production and spatial distribution. They have previously demonstrated that cGMP mediated activity varies depending on whether it is elevated via pGC or sGC, further supporting the compartmentalisation hypothesis. Most recently FRET imaging in live neonatal rat cardiac myocytes expressing cGMP and cAMP markers has shown that sGC mediated cGMP leads to activation of PKA-RI via cAMP, and a reduction in PKA-RII. These opposing effects are mediated via PDE2/3 regulation, which are confined to specific cellular locations also. Interestingly cAMP activity was not elevated via ANP/pGC signalling. In light of cAMP/PKA in regulating calcium transients and inotropic effects, these observations may highlight further therapeutic targeting of specific cGMP/cAMP as a result of spatially distinct cGMP/PDE signalling.

Conclusions

The evidence suggests that sGC/cGMP signalling to limit infarct size is a tractable therapeutic target; however it is clear that mass elevation of myocardial cGMP concentration in itself is not required to afford protection. Live cell imaging is now allowing real time visualisation of cGMP whilst exposing cells to pharmacological interventions. This information is required to ascertain how the dynamic signals mediate their action following differing stimuli, which cGMP elevating targets are important and which cGMP compartments may be key. With this information, suitable
cytoprotective interventions to limit the injurious effects of ischaemia-reperfusion injury via cGMP signalling could be developed.
Figure legends/Table headings

Figure 1
Structural representation of natriuretic peptide receptors (NPRs) and soluble guanylyl cyclases (sGCs) implicated in the production of cGMP. NB. NPR-C does not have particulate guanylyl cyclase activity and its mechanism of possible initiation of cGMP production remains to be eluded. sGC exists in two redox states that can catalyse cGMP production, however the oxidised form of sGC has no endogenous ligand.

Figure 2
Schematic representation of cytoprotective signalling cascades in cardiac myocytes incorporating cGMP. Included is the incorporation of NO$\textsubscript{3}^-$/NO$\textsubscript{2}^-$ as an alternative source/store of NO. Highlighted is the dynamic redox of sGC which is known to exist *in vivo*. Production of cGMP can be achieved by endogenous NO targeting the reduced sGC or exogenously by sGC stimulators. In addition sGC activators can target the oxidised state. Elevation of cGMP can also be achieved by targeting the membrane associated particulate guanylyl cyclase via the natriuretic peptide receptor ligands. cGMP is depicted in spatially distinct areas produced by different guanylyl cyclases and regulated by specific phosphodiesterases. Cytoprotective signalling via cGMP has been demonstrated to culminate in inhibition of the mPTP via reduced intracellular calcium concentrations and inhibition of mK$\textsubscript{ATP}$ channels.

Table 1
List of some of the important pharmacological agents that are able to positively modify cGMP concentrations.
Table 2

List of clinical trials (completed and ongoing) that utilise pharmacological agents that target NO/sGC/pGC/cGMP signalling to limit infarct size.


7. Rapoport RM, Murad F. Agonist-induced endothelium-dependent relaxation in rat thoracic aorta may be mediated through cGMP. *Circ Res*. 1983;52:352-357.


56. Cleeter MWJ, Cooper JM, Darley-Usmar VM, Moncada S, Schapira AHV. Reversible inhibition of cytochrome c oxidase, the terminal enzyme of the mitochondrial respiratory


Figure 1

NPR-A
ANP ≥ BNP >> CNP

NPR-B
CNP >> ANP ≥ BNP

NPR-C
ANP = CNP = BNP

Reduced sGC
NO >> CO

Oxidised sGC
no endogenous ligand

Domain
Ligand Binding
Kinase homology
Dimerisation
Guanylyl cyclase

Figure 1
<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Target/Action</th>
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<tbody>
<tr>
<td>NO donors (NOC-9, Spermine NONOate, S-NO-glutathione)</td>
<td>Direct NO donors which spontaneously release NO\textsubscript{X}</td>
<td>Act on sGC to catalyse production of cGMP</td>
</tr>
<tr>
<td>(\text{NO}_3^-) (GTN, Isosorbide mononitrate)</td>
<td>Classic nitro vasodilators which require metabolism to generate bioactive NO\textsubscript{X}</td>
<td>Metabolised products act on sGC to catalyse production of cGMP</td>
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<tr>
<td>(\text{NO}_2^-) (Sodium nitrite, amyl nitrite)</td>
<td></td>
<td></td>
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<tr>
<td>sGC stimulators</td>
<td>Novel group of compounds that target sGC independently of NO</td>
<td>Act at the haem site of sGC to catalyse production of cGMP</td>
</tr>
<tr>
<td>BAY-63-2521(Riociguat), BAY 41-2272</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sGC activators</td>
<td>Novel group of compounds that target sGC independently of NO and the sGC haem moiety</td>
<td>Act at site of sGC to catalyse production of cGMP</td>
</tr>
<tr>
<td>BAY 58-2667(Cinaciguat), BAY 60-2770, HMR 1766 (Ataciguat)</td>
<td></td>
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<tr>
<td>PDE5 inhibitors (Sildenafil, Vardenafil)</td>
<td>Phosphodiesterase inhibitors prevent breakdown of cGMP</td>
<td>Prevent phosphodiesterases hydrolysing cGMP</td>
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<tr>
<td>8-Br-cGMP, 8-pCPT-cGMP</td>
<td>Synthetic analogues of cGMP</td>
<td>Act as a selective activators of PKG</td>
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<tr>
<td>Natriuretic peptides (ANP, BNP, CNP)</td>
<td>Endogenous ligands for the NPR-A and NPR-B receptors</td>
<td>Activate NPR’s coupled to pGC to produce cGMP</td>
</tr>
<tr>
<td>Carbon monoxide-releasing molecules (CORMS)</td>
<td>CO carriers which release CO in the presence of biological stimuli</td>
<td>CO acts on sGC to catalyse the production of cGMP</td>
</tr>
<tr>
<td>Title</td>
<td>Centre/Lead Country</td>
<td>Start/End</td>
</tr>
<tr>
<td>---------------------------------------------------------------------</td>
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<td>-----------------</td>
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<tr>
<td>Nitric Oxide in Myocardial Infarction Size (NOMI)</td>
<td>INO Thearpeutics, USA</td>
<td>2006-2008</td>
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<tr>
<td>Randomized Placebo-Controlled Trial of Inhaled iNO in Acute ST-Segment Elevation MI Treated by Primary Angioplasty</td>
<td>TASMC, Israel</td>
<td>2009-2010</td>
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<tr>
<td>The Effects of Nitric Oxide for Inhalation on Myocardial Infarction Size</td>
<td>Universitaire Ziekenhuizen Leuven, Belgium</td>
<td>2010-2013</td>
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<tr>
<td>Nitrites in Acute Myocardial Infarction</td>
<td>University of Aberdeen, UK</td>
<td>2011-2014</td>
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<tr>
<td>Study Evaluating the Use of Vasoldilators to Reduce Infarct Size and Microvascular Obstruction in ST-Elevation MI</td>
<td>University Hospital Leicster, UK</td>
<td>2011-2014</td>
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