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REVIEW

The role of gasotransmitters NO, H_2S and CO in myocardial ischaemia/reperfusion injury and cardioprotection by preconditioning, postconditioning and remote conditioning

Ioanna Andreadou[1], Efstathios K Iliodromitis[2], Tienush Rassaf[3], Rainer Schulz[4], Andreas Papapetropoulos[1] and Péter Ferdinandy[5,6]

[1]*Faculty of Pharmacy, School of Health Sciences, University of Athens, Athens, Greece,* [2]*Second Department of Cardiology, Medical School, University of Athens, Attikon University Hospital, Athens, Greece,* [3]*Department of Medicine, Division of Cardiology, Pulmonary and Vascular Medicine, University Hospital Düsseldorf, Düsseldorf, Germany,* [4]*Department of Physiology, Justus-Liebig-University, Giessen, Germany,* [5]*Department of Pharmacology and Pharmacotherapy, Semmelweis University, Budapest, Hungary, and* [6]*Pharmahungary Group, Szeged, Hungary*

Correspondence
Ioanna Andreadou, Faculty of Pharmacy, School of Health Sciences, University of Athens, Panepistimiopolis, Zografou, Athens 15771, Greece. E-mail: jandread@pharm.uoa.gr; Péter Ferdinandy, Department of Pharmacology and Pharmacotherapy, Semmelweis University, Nagyvárad tér 4, Budapest 1089, Hungary. E-mail: peter.ferdinandy@pharmahungary.com

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Ischaemic heart disease is one of the leading causes of morbidity and mortality worldwide. The development of cardioprotective therapeutic agents remains a partly unmet need and a challenge for both medicine and industry, with significant financial and social implications. Protection of the myocardium can be achieved by mechanical vascular occlusions such as preconditioning (PC), when brief episodes of ischaemia/reperfusion (I/R) are experienced prior to ischaemia; postconditioning (PostC), when the brief episodes are experienced at the immediate onset of reperfusion; and remote conditioning (RC), when the brief episodes are experienced in another vascular territory. The elucidation of the signalling pathways, which underlie the protective effects of PC, PostC and RC, would be expected to reveal novel molecular targets for cardioprotection that could be modulated by pharmacological agents to prevent reperfusion injury. Gasotransmitters including NO, hydrogen sulphide (H_2S) and carbon monoxide (CO) are a growing family of regulatory molecules that affect physiological and pathological functions. NO, H_2S and CO share several common properties; they are beneficial at low concentrations but hazardous in higher amounts; they relax smooth muscle cells, inhibit apoptosis and exert anti-inflammatory effects. In the cardiovascular system, NO, H_2S and CO induce vasorelaxation and promote cardioprotection. In this review article, we summarize current knowledge on the role of the gasotransmitters NO, H_2S and CO in myocardial I/R injury and cardioprotection provided by conditioning strategies and highlight future perspectives in cardioprotection by NO, H_2S, CO, as well as their donor molecules.

LINKED ARTICLES

This article is part of a themed section on Pharmacology of the Gasotransmitters. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2015.172.issue-6

Abbreviations

3MP, 3-mercaptopyruvate; 3-MST, 3-mercaptopyruvate transferase; BCA, cyano-L-alanine; CBS, cystathionine β-synthase; CHD, coronary heart disease; CK, creatinine kinase; CORM, carbon monoxide-releasing molecule; CSE, cystathionine γ-lyase; CyPD, cyclophilin D; HPDTT, 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione; eNOS, endothelial nitric oxide synthase; FeTPPS, 5,10,15,20-tetrakis(4-sulphonatophenyl) porphyrinato iron; GYY4137, morpholin-4-ium 4-methoxyphenyl-morpholino-phosphinodithioate; HNO, nitroxyl; HO, haem oxygenase; I/R, ischaemia/reperfusion; iNOS, inducible nitric oxide synthase; L-NAME, N-nitro-L-arginine methylester; L-NNA, Nω-nitro-l-arginine; LV, left ventricular; MitoSNO, mitochondria-targeted *S*-nitrosothiols; mPTP, mitochondria permeability transition pore; nNOS, neuronal nitric oxide synthase; Nrf2, nuclear factor (erythroid-derived 2)-like 2; NSAIDs, non-steroidal anti-inflammatory drugs; $ONOO^-$, peroxynitrite; PRG, DL-propargylglycine; PC, preconditioning; PostC, postconditioning; RC, remote conditioning; RISK, reperfusion injury salvage kinase; ROS, reactive oxygen species; SAC, S-allylcysteine; SAFE, survivor activating factor enhancement; SOD, superoxide dismutase; XOR, xanthine oxidoreductase

Table of Links

TARGETS	LIGANDS
3-MST (MPST)	1400W
Akt	Allicin
Cystathionine γ-lyase (CSE)	CXCL12 (SDF-1α)
Cystathionine β-synthase (CBS)	
Guanylyl cyclase (GC)	Glyceryl trinitrate (nitroglycerin)
Haem oxygenase (HO)	NaHS
K_{ATP} ($K_{ir}6.x$) channels	L-NAME
L-type ($Ca_v1.2$) channels	Nicorandil
NO synthase (NOS)	Nitric oxide (NO)
PI3K	Pravastatin
Protein kinase G (PKG)	DL-Propargylglycine (PRG)

This Table lists the 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 the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a,b).

Introduction: cardioprotection and gasotransmitters

Ischaemic heart disease is one of the leading causes of mortality and morbidity in the industrialized societies. Therefore, therapeutic strategies to protect the ischaemic myocardium have been extensively studied. Ischaemic preconditioning (PC), postconditioning (PostC) and remote conditioning (RC) of myocardium are well-described adaptive responses in which brief exposure to ischaemia/reperfusion (I/R) prior to ischaemia (PC), at the immediate onset of reperfusion (PostC) or in a remote organ prior to, during or at reperfusion after sustained ischaemia (RC), respectively, leads to cardioprotection characterized by reduction of infarct size and occurrence of arrhythmias, and attenuation of cardiac dysfunction. Although the cardioprotective effect of conditioning strategies have been proven in several species including humans, it seems that the presence of cardiovascular risk factors, co-morbidities and their medications may interfere with cardioprotective signalling pathways (for extensive reviews, see Ferdinandy *et al.*, 2007; Ovize *et al.*, 2010; Hausenloy *et al.*, 2013; Ferdinandy *et al.*, 2014). The cellular mechanism of cardioprotective pathways are not exactly known, although several signal transduction cascades have been suggested as reviewed elsewhere (Ferdinandy *et al.*, 2007; Heusch *et al.*, 2008; Ovize *et al.*, 2010; Hausenloy *et al.*, 2013; Heusch, 2013). Better understanding of the underlying signal transduction of ischaemic conditioning strategies may provide an important paradigm for cardioprotection and their translation to clinical use of pharmacological interventions (Hausenloy *et al.*, 2013; Heusch, 2013). Various ligands occupy the specific surface receptors and then the cardioprotective modalities start with intracellular signalling transduction, which among others includes redox signalling by reactive oxygen species (ROS), S-nitrosylation by NO and its derivatives, S-sulphydration by hydrogen sulphide and O-linked glycosylation with β-N-acetylglucosamine. All these modalities interact and regulate an entire pathway, thus influencing each other. For instance, enzymes can be phosphorylated and/or nitrosylated in specific and/or different site(s), with consequent increase or decrease in their specific

activity. The cardioprotective signalling pathways are thought to converge on mitochondria, and various mitochondrial proteins have been identified as targets of these post-transitional modifications (see Heusch *et al.*, 2008; Pagliaro *et al.*, 2011).

Gasotransmitters are a growing family of regulatory molecules involved in multilevel regulation of physiological and pathological functions in mammalian tissues. It is now widely recognized that the gasotransmitters NO, along with hydrogen sulphide (H_2S) and carbon monoxide (CO), are involved in a multitude of physiological functions (Caliendo *et al.*, 2010; Szabo, 2010; Peers and Steele, 2012). In the cardiovascular system, the regulatory role of NO and H_2S includes vasorelaxation, stimulation of angiogenesis and cardioprotection (Szabo, 2010; Coletta *et al.*, 2012), and that of CO includes relaxation of coronary vascular smooth muscle and cardioprotection (Muchova *et al.*, 2007).

The synthesis and major metabolic pathways of NO are described in detail elsewhere (Moncada *et al.*, 1997; Pacher *et al.*, 2007; Rassaf *et al.*, 2014), including the current Themed Issue (see Csonka *et al.*, 2015). In brief, NO is produced in most of the mammalian tissues and cells by both enzymic and non-enzymic reactions. Three isoforms of NOS have been described, neuronal (nNOS), endothelial (eNOS) and inducible (iNOS) isoforms. NOS activity is regulated by compartmentalization, substrate and co-factor availability, endogenous inhibitors, as well as transcriptional, post-transcriptional and post-translational modulations. The formation of NO by NOS-independent enzymic and non-enzymic reduction of nitrite/nitrate from dietary and endogenous sources becomes especially important during ischaemia when pH becomes acidic and oxygen-dependent NOS activity is limited. The major biological reactions of NO includes oxidation to nitrite and nitrate as well as its reaction with superoxide to yield peroxynitrite anion ($ONOO^-$), a reactive nitrating and nitrosating agent. Important molecular targets of NO include metalloenzymes such as soluble guanylate cyclase (GC), haemoglobin and cytochromes, along with S-nitros(yl)ation of thiols yielding S-nitrosothiols (see Ferdinandy and Schulz, 2003; Pacher *et al.*, 2007; Tennyson and Lippard, 2011; Radi, 2013).

H_2S is generated from endogenous sources and is physiologically present in blood and other tissues. Endogenous H_2S generating enzymes have been identified in mammals. Desulphydration of cysteine is the major source of H_2S in mammals and is catalysed by the *trans*-sulphuration pathway enzymes, cystathionine β-synthase (CBS), cystathionine γ-lyase (CSE) and 3-mercaptopyruvate sulphurtransferase (3-MST). Cystathionine can be converted by CSE to form H_2S. CBS can form cystathionine from serine and homocysteine, and additionally can form H_2S from cysteine. Cysteine, along with α-ketoglutarate, is converted into 3-mercaptopyruvate (3MP) by cysteine aminotransferase. 3MP can then be broken down by 3-MST to form H_2S (Predmore *et al.*, 2012). In the heart, there is little CBS, whereas CSE is plentiful (Chen *et al.*, 1999; Geng *et al.*, 2004). The observation that the heart contains significant levels of H_2S synthesizing enzyme suggests that it represents an important source of H_2S generation (Szabo *et al.*, 2011). Intracellular H_2S is apparently rapidly oxidized to $S_2O_3^{2-}$ (thiosulphate) by mitochondria with the subsequent conversion into SO_3^{2-} and SO_4^{2-}. SO_3^{2-} and SO_4^{2-} are also produced upon oxidation of H_2S by activated neutrophils, where SO_3^{2-} induced the respiratory burst leading to further H_2S oxidation and loss by several endogenous oxidant species elevated during disease processes, such as NO, superoxide, hypochlorite, H_2O_2 and $ONOO^-$. Furthermore, SO_3^{2-} readily undergoes hepatic metabolism forming SO_4^{2-} (see Whiteman *et al.*, 2011). The physiological functions of H_2S are mediated by different molecular targets, such as different ion channels and signalling proteins. Alternations of H_2S metabolism lead to an array of pathological disturbances in the form of hypertension, atherosclerosis, heart failure, diabetes, cirrhosis, inflammation, sepsis, neurodegenerative disease, erectile dysfunction and asthma (see Lynn and Austin, 2010; Wang, 2012).

Endogenous CO is generated from endogenous sources and is now established as an important, biologically active molecule. CO is generated by haem oxygenases (HO-1 and HO-2) as a result of the degradation of haem. The catalysed reaction results in the formation of ferrous iron (Fe^{2+}), CO and biliverdin, which is rapidly reduced to bilirubin, a reaction that requires O_2 and NADPH. This reaction is biologically important as it is crucial to iron and bile metabolism, and also generates a highly effective antioxidant, bilirubin. Both atrial and ventricular cardiac myocytes express HO-1 and HO-2, and as in many other tissues, HO-1 expression is inducible (it is also known as heat shock protein 32), whereas HO-2 expression is constitutive (for a review, see Peers and Steele, 2012).

CO is capable of modulating a number of signalling pathways. These pathways include those involving NO/GC, ROS and MAPKs. The relevant biosynthetic enzyme, HO, has a central role in cellular antioxidant defence and vascular protection, and it may mediate many of the actions of drugs used in cardiovascular therapy (Muchova *et al.*, 2007). Cardiovascular tissues express HO, which metabolizes haem to form CO. Up-regulation of HO-1 occurs in the heart after stress such as I/R and provides cardioprotection; most evidence indicates that CO is responsible for most of these beneficial effects (Johnson *et al.*, 2004; Peers and Steele, 2012), as it exerts anti-apoptotic and cytoprotective effects (Stein *et al.*, 2012). CO also has antihypertensive and anti-inflammatory effects (Muchova *et al.*, 2007).

Besides the NO, H_2S and CO, there are other gasotransmitters, such as hydrogen, methane, as well as some noble gases (He, Xe) that also exert cardioprotective effects. Inhalation of hydrogen gas has been shown to limit infarct size following I/R injury in rat and in canine hearts via opening of mitochondrial K_{ATP} channels followed by inhibition of mitochondria permeability transition pore opening (mPTP) (Yoshida *et al.*, 2012). The noble gas helium (He) is capable of inducing early and late PC at concentrations of 70 and 30%, respectively, by prevention of mPTP opening (Pagel *et al.*, 2007; Huhn *et al.*, 2009). However, the majority of research conducted to date has examined the cardioprotective effects of xenon because this noble gas exerts anaesthetic and analgesic effects under normal (as opposed to hyperbaric) atmospheric pressure conditions. A growing body of experimental evidence indicates that brief, intermittent exposure to this noble gas before prolonged coronary artery occlusion and reperfusion protects against irreversible ischaemic injury (see Pagel, 2010). In this review, we will focus on NO, H_2S and CO.

The purpose of the present review is to summarize recent findings on the role of the gasotransmitters NO, H_2S and CO in myocardial I/R injury and cardioprotection and to highlight future perspectives in developing modulators of these gasotransmitters for cardioprotection.

NO in I/R injury and cardioprotection

The role of NO and peroxynitrite in I/R injury and in cardioprotection by PC has been extensively reviewed earlier (Ferdinandy and Schulz, 2003; Schulz *et al.*, 2004; Cohen *et al.*, 2006; Ferdinandy, 2006; Jones and Bolli, 2006). Therefore, here we focus on more recent studies and especially the involvement of NO signalling in PostC and RC that has not yet been reviewed.

Endogenous NO *in I/R injury and preconditioning*

Since the discovery of NO, abundant data have been accumulating on the role of NO in the signalling mechanism of I/R injury and cardioprotection by PC in the heart. In brief, NO is a well-known cardioprotective molecule via the cGMP/PKG pathway, as a key molecule in the RISK (reperfusion injury salvage kinase) and SAFE (survivor activating factor enhancement) cardioprotective pathways, and via S-nitrosylation of proteins including, for example, the sarco/endoplasmic reticulum Ca^{2+}-ATPase and several mitochondrial proteins (see Burley *et al.*, 2007; Heusch *et al.*, 2008; Sun and Murphy, 2010; Murphy *et al.*, 2012; Schulz and Ferdinandy, 2013). During ischaemia, there is an accumulation of tissue NO from both enzymic and non-enzymic sources; therefore, upon reperfusion, due to a burst of ROS release, NO is converted into peroxynitrite, thereby contributing to reperfusion injury. High doses of NOS inhibitors given before ischaemia decrease I/R injury via decreasing peroxynitrite formation. On the contrary, PC by brief periods of I/R cycles leads to moderate increase in NO and peroxynitrite formation, which, in turn, leads to a decrease in NO and peroxynitrite formation after a prolonged I/R, thereby leading to cardioprotection (Ferdinandy and Schulz, 2003; Schulz *et al.*, 2004; Ferdinandy, 2006). Indeed, in the presence of NOS inhibitors or in NO-deficient states, such as hyperlipidaemia, diabetes and sensory neuropathy, the cardioprotection afforded by PC is lost, showing that intact basal NO synthesis in the heart is necessary to achieve cardioprotection by PC (see Ferdinandy *et al.*, 2007).

The role of NO in late PC is still not precisely known. Although increased iNOS expression seems to be an important element of cardioprotection by late PC (for reviews, see Ferdinandy and Schulz, 2003; Jones and Bolli, 2006), it has been shown that late PC-induced iNOS expression does not lead to increased NO formation in the rat heart (Bencsik *et al.*, 2010).

In summary, although NO is an important element in triggering the signal of PC as well as in several cardioprotective signalling cascades, excess NO accumulation during ischaemia contributes to reperfusion injury via nitrative stress by peroxynitrite.

Endogenous NO in postconditioning

Endogenous NO and peroxynitrite have been shown by several studies to be involved in the mechanism of ischaemic PostC. In mouse isolated hearts, ischaemic PostC reduced the infarct size, the effect being blocked by treatment with the eNOS inhibitor L-NAME (Tong *et al.*, 2014). In rat hearts, ischaemic PostC increased cardiac peroxynitrite formation; however, PostC in the presence of the peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(4-sulphonatophenyl) porphyrinato iron (FeTPPS) inhibited the infarct size, reducing the effect of PostC (Kupai *et al.*, 2009). Similarly, intravenous FeTPPS, given before PostC, abolished its beneficial effect, as shown in another study (Li *et al.*, 2013). This study suggests that interaction of NO and ROS at early stages of reperfusion contributes to the triggering signal for cardioprotection by PostC. However, at late stages of reperfusion, ischaemic PostC reduced post-ischaemic myocardial iNOS activity and nitrotyrosine formation and reduces myocardial infarct size in rats and humans as well. Administration of the iNOS inhibitor 1400W mimicked, whereas 3-morpholinosydnonimine abolished the effects of PostC (Fan *et al.*, 2011). Thus, an increased NO-peroxynitrite signalling is important in triggering cardioprotection by PostC, which, in turn, reduces peroxynitrite-induced nitro-oxidative stress at late reperfusion, thereby contributing to cardioprotection.

In mouse hearts, ischaemic PostC reduces the infarct size independent of whether or not PKG is present, as shown in PKG knockout mice. Similarly, mitochondria-targeted *S*-nitrosothiols (MitoSNO), which accumulate within the mitochondria where they generate NO and carry out the S-nitrosation of thiol proteins, also reduce infarct size when given during reperfusion, independent of the presence of PKG (Methner *et al.*, 2013). MitoSNO protects mice hearts *in vivo* against I/R injury through *S*-nitrosation of mitochondrial complex I, which is the entry point for electrons from NADH into the respiratory chain. Reversible S-nitrosation of complex I slows down the reactivation of mitochondria during the crucial first minutes of the reperfusion of ischaemic tissue, thereby decreasing ROS production, oxidative damage and tissue necrosis. Inhibition of complex I is afforded by the selective S-nitrosation of Cys^{39} on the ND3 subunit, which becomes susceptible to modification only after ischaemia. These results indicate that rapid complex I reactivation contributes to I/R injury (Chouchani *et al.*, 2013). Apart from the respiratory complexes, ischaemic PostC causes a 25% or greater increase in S-nitrosylation (SNO) of a number of proteins, which is blocked by treatment with L-NAME in parallel with the loss of protection. Furthermore, 77 unique proteins with SNO sites only affected by PostC have been identified (Tong *et al.*, 2014).

While ischaemic PostC protection involves NO, in rats treated with nitroglycerin for 3 days to induce vascular nitrate tolerance that causes systemic nitro-oxidative stress, ischaemic PostC failed to decrease infarct size. Phosphorylation of ERK1/2, Akt or eNOS showed no significant differences in hearts being responsive to PostC or lacking protection due to nitrate tolerance (Fekete *et al.*, 2013).

In conclusion, it seems that an increased NO-peroxynitrite signalling is important in triggering cardiopro-

tection by PostC; however, PostC, in turn, will reduce peroxynitrite-induced nitro-oxidative stress at late reperfusion, thereby contributing to cardioprotection. The most important downstream signalling pathway for NO in PostC likely involves S-nitrosylation of proteins and is independent of cGMP signalling.

Endogenous NO in remote conditioning

There are limited results available so far, and they are somewhat controversial regarding the role of NO in RC. In an early study in a rat model of RC, induced by ischaemia of the small intestine, NOS inhibition by Nω-nitro-L-arginine (L-NNA) did not affect cardioprotection (Petrishchev *et al.*, 2001). Similarly, in rabbits preconditioned by pulmonary ischaemia, the NOS inhibitor L-NAME did not affect cardioprotection (Tang *et al.*, 2014). However, in rats with brief femoral artery ischaemia-induced myocardial PC, cardioprotection was mediated by a combination of increased NO synthesis, opening of mitoK_{ATP} channels and increased ROS production (Shahid *et al.*, 2008). In a rabbit renal ischaemia-induced remote PC, cardioprotection was associated with a PPAR-mediated increase in iNOS expression (Lotz *et al.*, 2011). In rabbits preconditioned by transient limb ischaemia, interestingly, pre-treatment by the NO donor S-nitroso-N-acetylpenicillamine abolished cardioprotection (Steensrud *et al.*, 2010). In a hind limb ischaemia-induced late PC mice model, cardioprotection was associated with increased iNOS expression (Li *et al.*, 2004).

In summary, the role of NO in RC is controversial, probably due to different methods to induce RC. The role of peroxynitrite in RC has not been studied yet.

Exogenous NO in cardioprotection

As NO is a cytoprotective molecule, NO donor drugs including NO gas itself are promising tools for pharmacological cardioprotection. In this section, NO donor therapies with potential cardioprotective effect are reviewed.

Organic nitrates. Organic nitrates are the oldest NO donor compounds. Glyceryl trinitrate (commonly called nitroglycerin) have been used for the prevention and treatment of ischaemic heart disease for more than 100 years. Organic nitrates effectively alleviate the severity of myocardial ischaemia via their haemodynamic effects, but also contribute to cardioprotection via a NO-induced activation of K_{ATP} channels in the heart (see Csont and Ferdinandy, 2005). However, the main limitation of long-term prophylactic nitrate therapy is the development of vascular nitrate tolerance, which leads to the attenuation of clinical efficacy (see Csont and Ferdinandy, 2005; Csont, 2010; Münzel and Gori, 2013). In preclinical studies, nitrate tolerance aggravated I/R injury and abolished the cardioprotective effect of PC, possibly due to increased systemic formation of peroxynitrite (see Ferdinandy *et al.*, 2007). A human study (Gori *et al.*, 2010) reported that the endothelial preconditioning effect of a single dose of nitroglycerin was lost upon a prolonged exposure to nitroglycerin. Nevertheless, the acute administration of nitrates appears not to interfere with RC in patients undergoing coronary artery bypass graft surgery (Kleinbongard *et al.*, 2013).

In conclusion, organic nitrates are effective cardioprotective agents; however, when nitrate tolerance develops, in addition to the loss of their cardioprotective effect, they may interfere with endogenous cardioprotective mechanisms via pathologically increased nitro-oxidative stress.

Nitrite. For a long time, it has been proposed that nitrite is just an inert metabolite of NO. Over the past years, however, it has been shown that nitrite can be recycled to bioactive NO under conditions of hypoxia/ischaemia via reaction with haemoglobin and other endogenous nitrite reductases such as myoglobin, neuroglobin, cytoglobin, xanthine oxidoreductase (XOR), eNOS and some mitochondrial enzymes (see Rassaf *et al.*, 2014). Therefore, modification of endogenous nitrite levels by exogenous nitrite and nitrate either from dietary sources or nitrite-containing preparations, as a therapeutic tool to modify NO signalling has been intensively investigated (see Rassaf *et al.*, 2014). The administration of sodium nitrite exerts cytoprotective effects in myocardial I/R injury (Webb *et al.*, 2004; Dezfulian *et al.*, 2007; Hendgen-Cotta *et al.*, 2008). Nitrite is reduced to NO, S-nitrosothiols, N-nitrosamines and iron-nitrosylated haem proteins during early reperfusion (Rassaf *et al.*, 2007). The cytoprotective effects of nitrite are independent of eNOS. Whereas Webb *et al.* showed that the reduction of nitrite to NO was XOR-dependent (Webb *et al.*, 2004), another research group demonstrated that myoglobin is the main nitrite reductase in the myocardium (Hendgen-Cotta *et al.*, 2008; 2010a,b; Totzeck *et al.*, 2012a,b), as the reduction in infarct size following administration of nitrite was completely abolished in myoglobin knockout mice. Two distinct mechanisms have been described for the protective effects of nitrite. In one mechanism, nitrite modifies and inhibits complex I by post-translational S-nitrosation. This dampens electron transfer and reduces ROS generation and ameliorates oxidative inactivation of complexes II–IV and aconitase. This prevents mPTP opening and cytochrome *c* release. The other potential mechanism of nitrite-induced protection relates to the modification of the mPTP opening. This plays a critical role in mediating cell death during I/R injury. Cyclophilin D (Cyp D), which accelerates mPTP opening, undergoes S-nitrosylation on Cys^{203}, leading to reduced mPTP opening in mice wild-type fibroblast but not in Cyp D knockout fibroblasts (Nguyen *et al.*, 2011).

In conclusion, a low dose of nitrite anion is a promising cardioprotective agent, at least in part, via its reduction to NO in the ischaemic heart.

Miscellaneous NO donors. Several pharmacological compounds directly stimulating NO signalling pathways have been demonstrated to protect the heart against I/R injury when applied before ischaemia or at reperfusion (see Jones and Bolli, 2006; Pacher *et al.*, 2007).

NO gas inhalation during coronary occlusion has been shown to provide infarct size reduction and leads to a decrease in peroxynitrite formation in rats and mice (Nagasaka *et al.*, 2008; Neye *et al.*, 2012; Shinbo *et al.*, 2013), showing that NO inhalation may represent a promising early intervention in acute myocardial infarction patients. Accordingly, a phase 2 clinical trial investigating the effects of NO

for inhalation in myocardial infarct size (NOMI trial, NCT01398384) is ongoing.

Several NO-releasing derivatives of known drugs have been developed and investigated for cardioprotection. In mice, a NO-releasing pravastatin (Ncx-6550) dose-dependently reduced infarct size following —I/R and the dose required for protection was one-tenth of that of pravastatin alone (Di Filippo *et al.*, 2010). More interestingly, however, pravastatin, in contrast to the same dose of simvastatin or ischaemic PostC, reduced infarct size in hypercholesterolaemic rabbits independent of its lipid lowering action, potentially through eNOS activation and attenuation of nitro-oxidative stress (Andreadou *et al.*, 2012). Nitro-aspirin (NCX4016) has also been shown to effectively reduce infarct size in preclinical models (Wallace *et al.*, 2002; Fu *et al.*, 2007). Nicorandil, a NO-donor and K_{ATP} channel opener, has been shown to reduce the infarct size in rabbit hearts (Argaud *et al.*, 2009). However, a meta-analysis of available small-scale clinical trials could not show direct benefit in myocardial infarction patients from nicorandil treatment, possibly due to the lack of large clinical trials (Wu *et al.*, 2013).

There are several novel compounds with potential cardioprotective properties, whose mechanisms of action depend upon NO signalling. PostC with isoflurane decreases infarct size in wild-type mice. Mitochondria isolated from postconditioned hearts require significantly higher *in vitro* calcium loading than did controls to open the mPTP. In hearts from eNOS knockout mice, isoflurane PostC failed to alter the infarct size or mPTP opening (Ge *et al.*, 2010). The mechanism(s) involved in modifying mPTP opening might involve indirect effects through protein kinases or direct SNO-protein modifications. Indeed, pharmacological PostC with diazoxide induced a redox-sensitive phosphorylation/translocation of Akt, ERK1/2 and glycogen synthase kinase 3β (GSK3β) into the mitochondria and increased mitochondrial S-nitrosylated proteins, such as voltage-dependent anion channels, in rat isolated hearts (Penna *et al.*, 2013). Moreover, a mitochondria-selective S-nitrosating agent, MitoSNO, has been shown to reduce the infarct size in mice (Chouchani *et al.*, 2013). In mouse isolated hearts, netrin-1 PostC reduced the infarct size and this effect is abolished by the NO scavenger, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (Bouhidel *et al.*, 2014). Apelin-13 was ineffective in reducing infarct size in rat isolated hearts with pre-treatment, but when administered after ischaemia, it reduced infarct size, which was partially blocked by LNNA. Thus, apelin-13 protects the heart only if given after ischaemia, and in this protection, NO plays an important role (Rastaldo *et al.*, 2011).

There is increasing evidence for the formation of the nitroxyl anion (NO^-, which exists as HNO in aqueous solutions) by NOS (Hobbs *et al.*, 1994), lending further support to the assumption that this redox sibling of NO is involved in modulation of cardiac function under both normal and pathological (post-ischaemic) conditions. The infusion of HNO, generated by the HNO donor Angeli's salt (sodium trioxodinitrate, $Na_2N_2O_3$), prior to I/R exerts protective effects in rat isolated perfused hearts, a protection that resembles the phenomenon of 'early preconditioning' (Pagliaro *et al.*, 2003). Furthermore, the vasoprotective effects of the HNO donor isopropylamine NONOate (IPA/NO) have been maintained in hypercholesterolaemia, and thus, HNO donors may represent future novel treatments for vascular diseases (Bullen *et al.*, 2011).

In conclusion, NO donor molecules and molecules that activate cardioprotective NO-dependent signalling pathways are promising tools for cardioprotection.

Role of endogenous H_2S in I/R injury and cardioprotection

H_2S in I/R injury

Endogenous H_2S may play a role in the regulation of cardiovascular function and inflammatory/immune responses as a potential endogenous gasotransmitter. Although the heart expresses all three H_2S generating enzymes, most of the work has focused on the role of CSE-derived H_2S. Most of the studies that investigated the role of endogenous H_2S in cardioprotection have used DL-propargylglycine (PRG) or cyano-L-alanine (BCA) as inhibitors for H_2S synthesis. It should be noted that these compounds exhibit selectivity towards CSE, allowing H_2S production to continue through CBS and 3-MST; moreover, they are known to inhibit other pyridoxal 5′-phosphate-dependent enzymes (Asimakopoulou *et al.*, 2013). In rat isolated hearts, infarct size increased when endogenous H_2S production was inhibited by blocking CSE with PRG (Bliksoen *et al.*, 2008). In the same experimental model, exogenous L-cysteine administration limited I/R injury through a mechanism that appeared to be at least partially dependent upon H_2S synthesis and production was attenuated by PRG treatment (Elsey *et al.*, 2010). Additionally, the modulation of endogenously produced H_2S by cardiac-specific overexpression of CSE significantly limited the extent of injury (Elrod *et al.*, 2007). Very recently, it has been shown that, in mice lacking CSE, myocardial I/R injury was exacerbated, whereas H_2S therapy attenuated I/R injury (King *et al.*, 2014).

H_2S in preconditioning. The endogenous production of H_2S is required for ischaemic PC. H_2S production was decreased when ventricular myocytes were subjected to ischaemia. PC significantly attenuated the inhibitory effect of ischaemia on H_2S production, proving that endogenous H_2S plays an important role in cardioprotection (Bian *et al.*, 2006). In rat isolated cardiac myocytes, CSE inhibition, using PRG or BCA, reversed the cardioprotective effects of ischaemic PC on cell viability and morphology (Pan *et al.*, 2006). Furthermore, treatment of cardiac myocytes with either PRG or BCA markedly decreased endogenous H_2S production and significantly attenuated the protective effect of PC (Bian *et al.*, 2006).

In an *in vivo* rat model of myocardial I/R, NaHS (a donor of H_2S) reduced infarct size, apoptosis, the expression levels of Fas, FasL and cleaved caspase-3 proteins. In contrast, PRG showed opposite effects to NaHS (Yao *et al.*, 2012). PRG administration for 1 week and 2 days after I/R abolished the decrease of infarct size, compared with the group treated with NaHS, whereas a marked reduction of the infarct size and up-regulation of survivin was observed in the group treated with NaHS (Zhuo *et al.*, 2009).

Table 1

The beneficial effects of endogenous H_2S in ischaemia/reperfusion injury, in PC and PostC

Experimental model	Effect of H_2S	Proposed mechanism(s)	Reference
Rat isolated hearts	L-cysteine reduced I/R in a dose-dependent manner and PRG reversed this protection	L-cysteine produced a threefold elevation of endogenous left ventricular H_2S concentration, and it was attenuated by PRG treatment	Elsey *et al.* (2010)
Rat cardiomyocytes; rat isolated hearts	PRG or BCA markedly decreased endogenous H_2S production and significantly attenuated the protective effect of PC	K_{ATP} and PKC activation	Bian *et al.* (2006)
Rat isolated ventricular myocytes	PRG or BCA reversed the cardioprotective effects of myocardial PC on cell viability, morphology and electrically induced $[Ca^{2+}]_i$	Activation of sarcolemmal K_{ATP} channels and/or provoking NO release	Pan *et al.* (2006)
Rat isolated hearts	PRG increased I/R	Decreased phosphorylation of Akt with PAG	Bliksoen *et al.* (2008)
Rat isolated hearts	Loss of PostC protection after PRG administration	Peak of H_2S production in the early reperfusion state	Huang *et al.* (2012)
Rat isolated hearts	PostC significantly stimulated H_2S synthesis enzyme activity during the early period of reperfusion. Administration of PRG abolished the protection of the PostC.	Activation of the pro-survival PKC-e and PKC-a	Yong *et al.* (2008)
In vivo (rats)	NaHS significantly reduced the myocardial infarct size. PRG administration showed opposite effects to NaHS. PRG increased I/R.	Reduced expression levels of Fas, FasL and cleaved caspase-3 proteins	Yao *et al.* (2012)
In vivo (rats)	PRG increased I/R	Up-regulation of survivin	Zhuo *et al.* (2009)
In vivo (mice overexpressing CSE in heart)	Reduction of reperfusion injury	Partial inhibition of mitochondrial respiration	Elrod *et al.* (2007)

H_2S in postconditioning. The endogenous production of H_2S is also required for ischaemic PostC. Indeed, ischaemic PostC stimulated the activity of H_2S-generating enzymes in the early phase of reperfusion (Yong *et al.*, 2008), and PRG partly attenuated the cardioprotective effect of PostC (Huang *et al.*, 2012). Pre-treatment with PRG, prior to global ischaemia, attenuated the reduction in infarct size by PostC (Yong *et al.*, 2008). Additionally, the modulation of endogenously produced H_2S by cardiac-specific overexpression of CSE significantly limited the extent of injury (Elrod *et al.*, 2007). The role of H_2S in RC has not yet been determined.

In conclusion, all the results described above demonstrate that H_2S may be of significant importance in the mechanism of cytoprotection during evolving myocardial infarction and that the modulation of endogenous production may be of clinical benefit in myocardial ischaemia. The beneficial effects of endogenous H_2S in I/R injury, in PC and PostC are summarized in Table 1.

Cardioprotection by exogenous H_2S administration

H_2S donors. By using exogenous H_2S therapy, the amount of injury is reduced in cardiomyocytes, in isolated *ex vivo* and in *in vivo* hearts of various models of I/R injury. The pharmacological modulation of H_2S is becoming a challenging field of research in drug discovery. The administration of gaseous H_2S is greatly limited by the difficulty to ensure an accurate control of dose and the risk of overdose (with serious consequences of H_2S toxicity). For the above reasons, the use of H_2S-releasing compounds seems to be the most convenient and satisfactory strategy (see Martelli *et al.*, 2012). NaHS is the prototypical example of a H_2S-generating agent, is a rapid H_2S donor and is widely used for experimental purposes. Ideal H_2S donors for therapeutic purposes should generate H_2S with slow releasing rates. This pharmacological feature seems to be exhibited by some natural derivatives. Indeed, the beneficial effects of garlic (*Allium sativum* L.) on cardiovascular functions have been well recognized for a long time. Alliin is a sulphur amino acid that is abundantly present in garlic and is converted to diallyl thiosulphinate (also known as allicin), which, in turn, rapidly decomposes to more stable organosulphur compounds, such as diallyl sulphide, ajoene and diallyl polysulphides (diallyl disulphide and trisulphide). Diallyl disulphide and trisulphide diallyl disulphide and trisulphide are true H_2S donors and release H_2S with a relatively slow mechanism, which requires the cooperation of endogenous thiols (such as reduced glutathione) (Benavides *et al.*, 2007). Besides the above-mentioned organic polysulphides

of natural origin, some synthetic H_2S-releasing agents described. Among them, the phosphinodithioate derivative GYY4137 (morpholin-4-ium 4-methoxyphenyl-morpholino-phosphinodithioate) represents an attractive example (Li *et al.*, 2008). Several concepts that have been previously used by medicinal chemistry for improving well-known drugs through the development of 'NO-hybrids' are presently being translated to the design of 'H_2S hybrids'. This general concept has been applied also to the design of H_2S-releasing non-steroidal anti-inflammatory drugs (NSAIDs), obtained through the conjugation of the 'parent' NSAIDs with a dithiolethione moiety [5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione] (HPDTT), which is currently the most widely used H_2S-releasing moiety for synthesizing pharmacological hybrids (Li *et al.*, 2007; Sparatore *et al.*, 2009). Another class of H_2S donors is thioamino acids that release H_2S upon reaction with bicarbonate at a rate that is faster than GYY4137, yet appreciably slower than Na_2S or NaSH (Zhou *et al.*, 2012). Detailed reviews of H_2S donors (Martelli *et al.*, 2012; Whiteman *et al.*, 2011; Kashfi and Olson 2013) and an excellent methodological review on how to measure H_2S (Nagy *et al.*, 2014) are available.

In conclusion, exogenous compounds, which behave as sources of H_2S, are viewed as powerful tools for basic studies and innovative pharmacotherapeutic agents for a variety of cardiovascular diseases.

In vitro *studies*. NaHS caused cardioprotection, in terms of cell viability and electrically induced calcium $[Ca^{2+}]_i$ transients (Pan *et al.*, 2006). In cultured cardiomyocytes, NaHS showed concentration-dependent inhibitory effects of apoptosis induced by hypoxia/reoxygenation (Yao *et al.*, 2010). Furthermore, NaHS significantly increased cell viability, percentage of rod-shaped cells and myocyte contractility (Hu *et al.*, 2008b). A rapid, time-dependent phosphorylation of JNK was observed in cultured rat neonatal cardiomyocytes, whereas NaHS inhibited this early phosphorylation of JNK, concluding that the early JNK inhibition during reperfusion is associated with H_2S-mediated protection against cardiomyocyte apoptosis (Shi *et al.*, 2009).

Perfusion with NaHS significantly improved post-ischaemic contractile recovery (Hu *et al.*, 2011), decreased myocardial infarct size and improved left ventricular (LV) contractile function (Bian *et al.*, 2006; Bliksoen *et al.*, 2008; Hu *et al.*, 2008a). Treatment of rat isolated hearts with NaHS, 10 min prior to the onset of coronary occlusion, resulted in a concentration-dependent limitation of infarct size (Johansen *et al.*, 2006).

The new H_2S-releasing derivative of diclofenac, S-diclofenac (2-[(2,6-dichlorophenyl)amino]benzene acetic acid 4-(3*H*-1,2-dithiole-3-thione-5-yl)-phenyl ester), had marked anti-ischaemic activity (Rossoni *et al.*, 2008). Furthermore, pharmacological PC with allitridum resulted in significantly smaller infarcts than in control hearts (Zhang *et al.*, 2001).

The mechanisms accounting for H_2S-induced cardioprotective activities in cardiomyocytes include prevention of leukocyte adherence (Zanardo *et al.*, 2006), inhibition of excessive production of NO and of NF-κB in macrophages (Oh *et al.*, 2006), activation of the sarcolemmal K_{ATP} channels (Pan *et al.*, 2006), deactivation of GSK-3β and decreased translocation of Bax, caspase-3 activation and inhibition of mPTP opening (Yao *et al.*, 2010). Moreover, H_2S produced delayed cardioprotection via K_{ATP}/PKC-dependent induction of COX-2 expression and via NO-induced COX-2 activation (Hu *et al.*, 2008b), inhibition of oxidative stress and activation of superoxide dismutase (SOD) (Sun *et al.*, 2012). The mechanism accounting for H_2S cardioprotective activities when it is administered prior to sustained ischaemia in isolated hearts involves a PI3K/Akt/PKG-dependent mechanism (Hu *et al.*, 2011), contribution of K_{ATP}/PKC/ERK1/2 and PI3K/Akt pathways (Hu *et al.*, 2008a), and expression of heat shock protein 72 (Bliksoen *et al.*, 2008).

However there is controversy over the involvement of mitoK_{ATP} channels in the cardioprotective activity of H_2S. Bian *et al.* (2006) showed that blockade of mitoK_{ATP} channels with 5-hydroxydecanoic acid had no effect on the cardioprotection afforded by exogenous H_2S, suggesting that contrary to the mechanism of classic PC, mitoK_{ATP} channels most probably do not play a major role in the cardioprotection afforded by H_2S. However, other studies (Johansen *et al.*, 2006; Rossoni *et al.*, 2008) have shown that pre-treatment with the K_{ATP} channel blockers glibenclamide or 5-hydroxydecanoate abolished the infarct-limiting effect of NaHS.

Concerning the effect of H_2S on PostC, studies in isolated hearts have shown that treatment with NaHS at the onset of reperfusion results in a reduction of infarct size (Luan *et al.*, 2012), a cardioprotective effect similar to that of PostC (Ji *et al.*, 2008). In another study, treatment with NaHS also resulted in a significant improvement in LV function and reduction of arrhythmia scores (Zhang *et al.*, 2007). Pharmacological PostC with six cycles of a 10 s infusion of NaHS or 2 min continuous NaHS infusion reduced myocardial infarct size in rat isolated hearts (Yong *et al.*, 2008).

In isolated hearts, it seems that mitoK_{ATP} channel opening is involved in the H_2S-induced PostC (Zhang *et al.*, 2007; Ji *et al.*, 2008). H_2S PostC confers the protective effects against I/R injury also through the activation of Akt, PKC and eNOS pathways (Yong *et al.*, 2008). Moreover, recently, it has been shown that H_2S PostC protected rat isolated hearts via the activation of the JAK2/STAT3 signalling pathway (Luan *et al.*, 2012).

In conclusion, further studies are required to elucidate the potential role of H_2S as a cytoprotective mediator against myocardial I/R injury, the mechanisms regulating its generation and the nature of its interaction with protein targets such as the K_{ATP} channels.

In vivo *studies*. NaHS administration before sustained ischaemia resulted in a remarkable reduction of the infarct size (Zhuo *et al.*, 2009) and significantly reduced cell apoptosis (Sivarajah *et al.*, 2009; Yao *et al.*, 2012). Furthermore, a single bolus of NaHS administered 24 h before myocardial infarction produced a strong infarct-limiting effect and a time-course study demonstrated that the protection lasted for at least 3 days after the PC stimulus (Pan *et al.*, 2009). When H_2S was administered to mice before myocardial ischaemia, it provided profound protection against ischaemic injury (Calvert *et al.*, 2009).

In the above studies, the mechanism of the cardioprotective effect of NaHS in the *in vivo* models was focused on anti-apoptotic and anti-inflammatory effects. H_2S reduced

calcineurin activity and the expression levels of Fas, FasL and cleaved caspase-3 proteins (Yao *et al.*, 2012). More specifically, during the early PC period, H_2S increased the nuclear localization of Nrf2, a transcription factor that regulates the gene expression of a number of antioxidants and increased the phosphorylation of PKCε and STAT-3. During the late PC period, H_2S increased the expression of antioxidants (HO-1 and thioredoxin 1), of heat shock proteins 90 and 70, Bcl-2, Bcl-xL and COX-2, and also inactivated the pro-apoptogen Bad (Calvert *et al.*, 2009). Furthermore, it attenuated the increase in caspase 9 activity, the decrease in the expression of Bcl-2, the phosphorylation of p38 MAPK and JNK, the polymorphonuclear leukocyte accumulation, myeloperoxidase activity, malondialdehyde levels, and nitrotyrosine staining in rat hearts, subjected to regional myocardial I/R. The cardioprotective effects of NaHS were abolished by 5-hydroxydeconoate; thus, it seems that the anti-apoptotic effect of NaHS may partially be related to the opening of the mitoK$_{ATP}$ channels (Sivarajah *et al.*, 2009). PC with H_2S also produced strong late cardioprotection through a PKC-dependent mechanism (Pan *et al.*, 2009). Studies to explore the molecular mechanism of H_2S-induced cardioprotection in mice showed that administration of NaHS increased significantly serum as well as myocardial NO levels without any sign of myocardial injury. Typical characteristics of isoprenaline-induced myocardial injury were abolished by NaHS administration as shown by reduction in elevated thiobarbituric acid reactive substances and normalization of GSH, glutathione peroxidase, SOD and catalase activity. Furthermore, a decrease in TNF-α expression and an improvement of myocardial architecture was also observed and the inhibition of NOS abolished the H_2S-induced cardioprotection in mice (Sojitra *et al.*, 2012).

S-allylcysteine (SAC), which is an organosulphur-containing compound derived from garlic, mediated cardioprotection via a H_2S-related pathway in rats and significantly lowered mortality and reduced infarct size, whereas protein expression studies revealed that SAC up-regulated CSE expression (Chuah *et al.*, 2007).

In anaesthetized and mechanically ventilated pigs subjected to ischaemia/reperfusion, Na_2S reduced the heart rate and the cardiac output without affecting stroke volume (Simon *et al.*, 2008). In animals with co-morbidities, administration of Na_2S beginning 24 h or 7 days before myocardial ischaemia significantly decreased infarct size in *db/db* diabetic mice. This result indicated that diabetes did not alter the ability of H_2S to increase the nuclear localization of Nrf2, but it impaired aspects of Nrf2 signalling. Exogenous administration of Na_2S attenuated myocardial ischaemia–reperfusion injury in *db/db* mice, suggesting the potential therapeutic effects of H_2S in treating lethal arrhythmias and heart attack in the setting of type 2 diabetes (Peake *et al.*, 2013).

However, few studies so far have investigated the cardioprotective effect of exogenous administered H_2S during reperfusion. The delivery of H_2S at the time of reperfusion can limit infarct size and preserve LV function in mice (Elrod *et al.*, 2007). In an experimental model of shock and ischaemia/reperfusion, haemorrhage-induced lactic acidosis and *ex vivo* vascular hyporeactivity, were attenuated by NaHS (Issa *et al.*, 2013). Post-therapeutic sulphide provided protection following I/R injury in pigs, improved myocardial function, reduced infarct size and improved coronary microvascular reactivity (Sodha *et al.*, 2009). The effects of different regimens of parenteral H_2S administration on myocardium during I/R were investigated in Yorkshire pigs. Continuous, but not bolus H_2S infusion markedly reduced myocardial infarct size and improved regional LV function, as well as endothelium-dependent and endothelium-independent microvascular reactivity (Osipov *et al.*, 2009).

The cytoprotection observed in these studies was mainly associated with an inhibition of myocardial inflammation and a preservation of both mitochondrial structure and function after I/R injury (Elrod *et al.*, 2007). NaHS protected against the effects of haemorrhage-induced I/R by acting primarily through a decrease in both pro-inflammatory cytokines and iNOS expression and an up-regulation of the Akt/eNOS pathway (Issa *et al.*, 2013). Exogenous sulphide may have therapeutic utility in clinical settings in which I/R injury is encountered potentially through its anti-inflammatory activities (Sodha *et al.*, 2009). The beneficial effects of exogenous H_2S administration in cardioprotection are shown in Table 2.

In conclusion, there are only few studies concerning the cardioprotective effects of exogenous administration of H_2S in models of I/R *in vivo*. The intracellular signalling pathways underlying the protection are completely unknown. Furthermore, there is no study of the role(s) of H_2S in triggering PostC *in vivo*.

Human studies. It has been suggested that modulating systemic H_2S production may represent a viable approach for the treatment of vascular disease. In one study comparing patients with coronary heart disease (CHD) with angiographically normal subjects, the number of affected coronary vessels correlated with decreased plasma levels of H_2S. More specifically, plasma levels of H_2S were significantly lower in CHD patients with coronary artery occlusion than in patients with simple stenosis and were also lower in hypertensive patients than normotensive ones (Jiang *et al.*, 2005).

A clinical study has been performed in order to evaluate the effectiveness of allicor (garlic powder tablets) treatment in primary CHD prevention and its effects on the estimates of multifunctional cardiovascular risk. A 12 month treatment with allicor resulted in the significant decrease of cardiovascular risk by 1.5-fold in men and by 1.3-fold in women, and the main effect that played a role in cardiovascular risk reduction was the decrease in low-density lipoprotein cholesterol (Sobenin *et al.*, 2010). The effect of 6 weeks of administration of garlic oil was observed on cardiac performance and exercise tolerance in 30 patients of CHD. Garlic significantly reduced heart rate at peak exercise and also reduced the workload upon the heart, resulting in better exercise tolerance as compared with the initial test (Verma *et al.*, 2005).

In conclusion, there are no clinical studies so far to confirm the cardioprotective role of H_2S in humans.

Role of endogenous CO in I/R injury and cardioprotection

The effect of severe hypoxia and reoxygenation on HO-1 expression has been investigated in cardiomyocytes and the potential protective role of HO-1 and its product bilirubin

Table 2

The beneficial effects of exogenous administered H_2S in ischaemia/reperfusion injury, in PC and PostC

Experimental model	Effect of H_2S	Proposed mechanism(s)	Reference
Rat isolated ventricular myocytes (NaHS)	Cardioprotection in terms of cell viability, morphology and electrically induced $[Ca^{2+}]_i$	Activation of sarcolemmal K_{ATP} channels and/or provoking NO release	Pan *et al.* (2006)
Cardiomyocytes (NaHS)	Reduction of apoptosis	Phosphorylation of GSK-3β (Ser^9) and subsequent inhibition of mPTP opening	Yao *et al.* (2010)
Isolated cardiac myocytes (NaHS)	Increased cell viability, percentage of rod-shaped cells, and myocyte contractility	Delayed cardioprotection via K_{ATP}/PKC-dependent induction of COX-2 expression and via NO-induced COX-2 activation	Hu *et al.* (2008b)
Primary cultured rat neonatal cardiomyocytes (NaHS)	Decreased the number of apoptotic cells, lowered cytochrome *c* release	Inhibition of the early phosphorylation of JNK, enhanced Bcl-2 expression	Shi *et al.* (2009)
Rat isolated hearts PC (NaHS)	Improved post-ischaemic contractile function	Suppression of NHE-1 activity involving a PI3K/Akt/PKG-dependent mechanism	Hu *et al.* (2011)
Rat isolated hearts PC (NaHS)	Decreased myocardial infarct size and improved heart contractile function	K_{ATP}/PKC/ERK1/2 and PI3K/Akt pathways	Hu *et al.* (2008a)
Rat isolated hearts PC (NaHS)	Reduction of infarct size	Activation of PKC and sarcK_{ATP}. No involvement of mitoK_{ATP}.	Bian *et al.* (2006)
Rat isolated hearts PC (NaHS)	Dose-dependent reduction of infarct size	Involvement of K_{ATP} channels.	Johansen *et al.* (2006)
Perfused rat hearts; NaHS was added to the perfusate during stabilization and throughout the experiment	Non-significant decrease in infarct size	Expression of heat shock protein 72	Bliksoen *et al.* (2008)
Rabbit isolated heart; H_2S-releasing derivative of diclofenac	Marked anti-ischaemic activity	Increased GSH formation leading to activation of K_{ATP} channels	Rossoni *et al.* (2008)
Rabbit isolated heart; allitridum PC	Decrease infarct size	Blocked by administration of Poly B, an inhibitor of PKC, implying that PKC has an important role in PC	Zhang *et al.* (2001)
Rat isolated hearts NaHS-PostC	Reduction of I/R. Reduction of CK.	K_{ATP} channels involvement	Ji *et al.* (2008)
Isolated hearts NaHS-PostC	Significant improvement in heart function and arrhythmia scores	H_2S increases the open probability of K_{ATP} in cardiac myocytes	Zhang *et al.* (2007)
Isolated hearts NaHS-PostC	Reduction of infarct size	Activation of the JAK2/STAT3 signalling pathway	Luan *et al.* (2012)
Isolated hearts NaHS PostC	Reduction of infarct size	Activation of AKT, PKC, eNOS	Yong *et al.* (2008)
In vivo (mice) NaHS PC	Reduction of infarct size, decrease of troponin-I	Decrease of oxidative stress, increase Nfr2, PKCε, STAT-3, HO-1, Trx1, HSP90, HSP70, Bcl-2, Bcl-xL, COX-2 and decrease of Bad	Calvert *et al.* (2009)
In vivo (rats) NaHS PC	Reduction of infarct size	Up-regulation of survivin	Zhuo *et al.* (2009)
In vivo (rats) NaHS PC	Reduction of infarct size	Reduction of calcineurin, Fas, Fas-L, caspase-3 and increase of ARC	Yao *et al.* (2012)
In vivo (rats) NaHS PC	Reduction of infarct size	PKC-dependent mechanism	Pan *et al.* (2009)
In vivo (rats) NaHS PC	Reduction of infarct size	Decrease in caspase-9, increase of Bcl-2, mitoK_{ATP} opening and increased phosphorylation of p38	Sivarajah *et al.* (2009)
In vivo rats S-allylcysteine (SAC) PC	Reduction of infarct size	SAC up-regulated CSE expression	Chuah *et al.* (2007)
In vivo (pigs) Na_2S PC	Reduction of infarct size	Lower lactate, improvement in noradrenaline response. No change in oxidative stress.	Simon *et al.* (2008)
In vivo (mice) NaHS-PostC	Dose dependent reduction of I/R.	Anti-inflammatory properties, preservation of mitochondrial function	Elrod *et al.* (2007)
In vivo (rat) model of haemorrhage-induced I/R NaHS PostC	Shock and I/R induced a decrease in MAP, lactic acidosis and *ex vivo* vascular hyporeactivity, which were attenuated by NaHS	Decrease in both pro-inflammatory cytokines and iNOS expression and an up-regulation of the Akt/eNOS pathway	Issa *et al.* (2013)
In vivo (pigs) NaHS-PostC	Reduction of infarct size	Anti-inflammatory effects	Sodha *et al.* (2009)
In vivo (pigs) NaHS-PostC	Reduction of infarct size	Markers of apoptosis and autophagy anti-apoptotic effects	Osipov *et al.* (2009)
db/db diabetic mice (Na_2S) PC	Decreased myocardial injury	Impair aspects of Nrf2 signalling	Peake *et al.* (2013)

against reoxygenation damage was assessed. Hypoxia caused a time-dependent increase in both HO-1 expression and HO activity, which gradually declined during reoxygenation which produced marked injury. However, incubation with haemin or bilirubin during hypoxia considerably reduced the damage that was developed during reoxygenation. Generation of ROS was enhanced after hypoxia, whereas haemin and bilirubin attenuated this effect, indicating that the HO-1-bilirubin pathway can effectively defend hypoxic cardiomyocytes against reoxygenation injury and highlight the importance of haem availability in the cytoprotective action afforded by HO-1 (Foresti *et al.*, 2001). Additionally, the cardioprotection obtained by gene delivery of the hypoxia-inducible factor, HIF-1α, depended upon the downstream factor HO-1. HIF-1α and HO-1 provided protection against H_2O_2-induced damage in HL-1 cells. Remote gene delivery of HIF-1α afforded cardioprotective effects, which were dependent upon HO activity, indicating that downstream to HO-1, bilirubin and CO may be organ effectors (Czibik *et al.*, 2009).

Moreover, HO-1-deficient mice develop right ventricular infarction after chronic hypoxia exposure and are more susceptible to I/R injury (Yet *et al.*, 1999; Yoshida *et al.*, 2001) and HO-1 overexpression protects the myocardium from I/R injury (Yet *et al.*, 2001).

Johnson *et al.* tested the hypothesis that cardiac HO-1 expression is increased in Dahl salt-sensitive (SD) rats with salt-induced hypertension: the rats were placed on a high- or low-salt diet for 4 weeks and cardiac HO isoform expression were determined in isolated paced Langendorff hearts. Coronary arterial HO-1 immunostaining was enhanced in high-salt rats and suggested that coronary HO-1 expression is increased to promote enhanced coronary vasodilatation in salt-induced hypertension (Johnson *et al.*, 2004).

One target of CO appeared to be the L-type Ca^{2+} channel. CO directly inhibited wild-type rat cardiomyocyte L-type Ca^{2+} currents and the recombinant α1C subunit of the human cardiac L-type Ca^{2+} channel (Scragg *et al.*, 2008). It also inhibited recombinant and native forms of this cardiac channel via mitochondria-derived ROS, actions likely to contribute to the protective effects of CO (Peers and Steele, 2012).

The interaction between the CBS/H_2S and HO-1/CO systems during myocardial I/R was also investigated in SD rats with hydroxylamine, a CBS inhibitor and zinc protoporphyrin (a HO-1 inhibitor). The H_2S, CO, GSH and SOD levels were decreased, the MDA level increased and the HO-1-mRNA and CBS-mRNA expression levels decreased, compared those in with rats subjected to I/R only, suggesting that both CBS/H_2S and HO-1/CO systems play a protective role in myocardial I/R and they interact with each other (Zhu *et al.*, 2008).

In conclusion, although the endogenous production of CO is required for ischaemic PC, the role of CO in PostC and RC has not been investigated yet.

Cardioprotection by exogenous administration of CO

CO donors. Several approaches have been used to investigate the therapeutic potential of CO, ranging from direct inhalation of CO gas to the use of prodrugs which then generate CO upon metabolism. A novel approach involves the use of specific CO carriers, which will release measurable, controllable and effective amounts of CO into biological systems. Transitional metal carbonyls based on iron, manganese or ruthenium have recently been developed as CO-releasing molecules (CORMs) that, under appropriate conditions, will release CO. The problem of low solubility of typical metal carbonyls has prompted the search for more biocompatible metal carbonyl complexes bearing amino acids (and their derivatives) as auxiliary ligands. The facial tricarbonyl fac-[RuCl(glycinate)(CO)3], often referred to as CORM-3, is the prototypical water-soluble CORM in this area (Chatterjee, 2004; Johnson *et al.*, 2007). Details of CO donors (Romão *et al.* 2012;, Zobi, 2013; Gonzales and Mascharak, 2014) and an excellent methodological review on measurement techniques of CO (Motterlini and Otterbein, 2010) are available. Such molecules confer cardioprotection both in *ex vivo* and *in vivo* experiments.

In conclusion, CORMs are an emerging class of pharmaceutical compounds that can be used in general consensus that the therapeutic effects elicited by these molecules may be directly ascribed to the biological function of the released CO.

In vitro *studies.* Pre-treatment with CO prevented apoptosis in cardioblastic H9c2 cells subjected to I/R. Reperfusion following brief periods of ischaemia induced cytochrome *c* release, activation of caspase-9 and caspase-3, and apoptotic nuclear condensation. Pre-treatment with CO or with the caspase-9 inhibitor (Z-LEHD-FMK) attenuated these apoptotic changes. Furthermore, I/R increased the phosphorylation of Akt after CO pretreatment, whereas the specific Akt inhibitor API-2 blunted the anti-apoptotic effect of CO, suggesting that CO induces mitochondrial generation of $O_2^{\bullet-}$, which is then converted by SOD to H_2O_2, and the subsequent Akt activation by H_2O_2 attenuates apoptosis during —I/R (Kondo-Nakamura *et al.*, 2010).

Increased cardiac expression of the chemokine CXCL12 (SDF-1α) promoted neovascularization and myocardial repair after ischaemic injury through recruiting stem cells and reducing cardiomyocyte death. CO gas and a CO-releasing compound, tricarbonyldichlororuthenium (II) dimer, dose-dependently induced CXCL12 expression in primary neonatal cardiomyocytes and H9C2 cardiomyoblasts. CO treatment enhanced neovascularization in the myocardium in the peri-infarct region and improved cardiac function. CO-mediated SDF-1α expression and Akt-dependent up-regulation of the transcription factor AP-2α is essential for CO-induced expression of CXCL12 and myocardial repair after ischaemic injury (Lin *et al.*, 2013). Furthermore, the anti-apoptotic behaviour of CO is attributed to the inhibition of mitochondrial membrane permeabilization, a key event in the intrinsic apoptotic pathway. In isolated non-synaptic mitochondria, CO partially inhibited loss of potential, mPTP opening, swelling and cytochrome *c* release (Queiroga *et al.*, 2010).

The most salient feature of CO-mediated cytoprotection is the suppression of inflammation and cell death. One of the important cellular targets of CO is the macrophage. Exposure of macrophages to CO results in the generation of an anti-inflammatory phenotype that leads to and preserves cellular homeostasis at the site of injury (Chin *et al.*, 2007).

Pretreatment of endothelial cells with CORM-2 resulted in the decrease of LPS-induced production of ROS and NO, up-regulation of HO-1, decrease in iNOS, inhibition of NF-κB

and downregulation of expression of intercellular adhesion molecule 1 (ICAM-1) (Sun *et al.*, 2008). Cardiac cells pre-treated with CORM-3 were more resistant to the damage caused by hypoxia-reoxygenation and oxidative stress. Cardioprotection was lost when CORM-3 was replaced by an inactive form (iCORM-3) that is incapable of liberating CO (Clark *et al.*, 2003). When interpreting data from studies using CORM-2 and CORM-3, it should be kept in mind that these agents while releasing CO, also release ROS (Marazioti *et al.*, 2011).

Concerning the effect of CO on PC, studies in isolated hearts have shown that exposure to CO alters or raises the ischaemic tolerance. PC accelerated the development of ischaemic contracture, increased the pre-ischaemic coronary flow and improved contractility, whereas CO exposure increased the baseline coronary flow and the contracture magnitude, improved both contractile recovery and ventricular arrhythmia incidence, and increased the hyperaemic coronary flow. Thus, CO-exposed hearts could be preconditioned in the same way as normal myocardium (Rochetaing *et al.*, 2001). When rat isolated hearts subjected to I/R and treated with CORM2, they exhibited significant reduction in post-ischaemic levels of the myocardial injury markers CK and LDH. Moreover, CORM-2 showed significantly improved post-ischaemic recovery of heart rate, coronary flow rate, cardiodynamic parameters and reduced infarct size (Soni *et al.*, 2012). PC with CORM-2 in rat isolated hearts markedly decreased LDH and CK levels in coronary effluent after global ischaemia, providing also a significant improvement in coronary flow rate, heart rate, cardiodynamic parameters and marked attenuation in infarct size (Soni *et al.*, 2010). In addition, marked LV dysfunction following coronary artery occlusion and reperfusion was ameliorated by CORM-3 in mouse hearts (Clark *et al.*, 2009).

The mechanism by which CORM-2 triggers PC in rat isolated hearts is probably due to the activation of the p38 MAPK β and PKC pathways before ischaemia as well as PI3-kinase pathway during reperfusion (Soni *et al.*, 2012) and activation of the K_{ATP} channels (Soni *et al.*, 2010).

Concerning the effect of CO on PostC, perfusion with CORM-2 during the first 10 min of reperfusion inhibited the release of LDH and CK, and reduced the infarct size in isolated hearts (Mei *et al.*, 2007). When isolated hearts were reperfused in the presence of CORM-3 after an ischaemic event, their myocardial performance was significantly improved and infarct size was reduced, whereas cardioprotection was lost when CORM-3 was replaced by an inactive form (iCORM-3) (Clark *et al.*, 2003). Cardioprotective effects of both CORM-2 and CORM-3 during reperfusion were probably mediated through activation of mitoK_{ATP} channels (Clark *et al.*, 2003; Mei *et al.*, 2007). Furthermore, cardioprotection by CO could also involve the NOS-cGMP and HO-1 pathways (Mei *et al.*, 2007).

In conclusion, although CO is essential to the triggering of PC and PostC in *in vitro* models of ischaemia/reperfusion, further studies are required to elucidate the mechanisms regulating its cardioprotective effects.

In vivo *studies*. CORM-3 induces delayed protection against myocardial infarction in an *in vivo* model of ischaemia/reperfusion. Pre-treatment with CORM-3 24 h prior to coronary occlusion markedly reduced infarct size, and the infarct-sparing effect of CORM-3 was still evident 72 h after administration of the CO donor, showing that CORM-3 induced delayed protection against myocardial infarction, which was similar to that afforded by the late phase of PC (Stein *et al.*, 2005). Using the same CO-releasing molecule as above, the same authors showed that pre-treatment with CORM-3 in mice resulted in a significant reduction in markers of apoptosis after I/R injury. CORM-3 triggered a cardioprotective signalling cascade that recruited the transcription factors NF-κB, STAT1/3 and Nrf2 with a subsequent increase in cardioprotective and anti-apoptotic molecules in the myocardium, leading to the late PC-mimetic infarct-sparing effects (Stein *et al.*, 2012).

However, only one study until now has investigated the cardioprotective effects of CO in a large animal model of I/R. Pre-treatment with low-dose CO 120 min before regional ischaemia and reperfusion did not provide acute protection as indicated by metabolic, energy-related and injury markers in pigs. The cardioprotective effects of CO either require higher doses or occur later after reperfusion (Ahlström *et al.*, 2011). The beneficial effects of endogenous CO and exogenous CO administration in cardioprotection are shown in Table 3.

In conclusion, there are only a limited number of studies regarding the cardioprotective effects of exogenous administration of CO in *in vivo* ischaemia–reperfusion models. Furthermore, to the best of our knowledge, there is no study that investigates the effect of CO in triggering PostC in an *in vivo* model of ischaemia/reperfusion.

Human studies. Endogenous CO at physiological concentrations is cytoprotective, whereas excess levels reflect underlying oxidative stress, inflammation and vascular pathology and indicate adverse clinical sequelae. However, the relation of exhaled CO to metabolic/vascular risk in the community is unknown (Cheng *et al.*, 2010). Most of the clinical studies up to now have investigated the role of CO as a poison in humans. Functionally, excess endogenous CO can lead to the formation of ROS, (Zhang and Piantadosi, 1992) can impair NO-mediated vasodilation (Durante, 2002) and can promote adverse vascular remodelling (Peyton *et al.*, 2002).

Although there are no clinical studies with drugs that liberate CO, we should mention that widely used drugs such as statins and fibrates have been shown to activate HO-1. HO-1 exerts multifunctional roles in the CVS, it cooperates with its downstream products, CO and bilirubin, to exert diverse cellular protective effects and provide potential therapeutic targets. Simvastatin has been shown to induce HO-1 in human smooth muscle cells (Lee *et al.*, 2004). Blocking HO-1 activity by zinc protoporphyrin or a small interfering RNA decreased the anti-inflammatory effect of simvastatin through inhibition of NO production, NF-κB activation and p21. The Akt and p38 MAPK pathways appeared to mediate the effect of simvastatin on HO-1 induction. This finding suggests that statins may provide a new therapeutic approach to the activation of HO-1.

Moreover, fenofibrate, rosiglitazone and troglitazone, which are ligands of the PPAR, also increase the expression of HO-1 in smooth muscle (Kronke *et al.*, 2007). Other pharmacological agents, such as curcumin, resveratrol, cyclosporine,

Table 3

The beneficial effects of endogenous and exogenous CO in ischaemia/reperfusion injury, in PC and PostC

Experimental model	Effect of CO	Proposed mechanism(s)	Reference
Cardiomyocytes (HO-1 expression) – treatment with haemin and bilirubin	Protective against reoxygenation damage	Attenuation of ROS	Foresti *et al.* (2001)
HL-1 cells – gene delivery of HIF-1α	Cardioprotection	Depended upon HO activity indicating that downstream to HO-1, bilirubin and CO may be organ effectors	Czibik *et al.* (2009)
HO-1 overexpression	Protects the myocardium from ischaemic and reperfusion injury		Yet *et al.* (2001)
Dahl salt-sensitive (SD) rats	Cardioprotection by promoting coronary vasodilatation	HO-1 expression is increased	Johnson *et al.* (2004)
H9c2 cells (CO)	Prevention of apoptosis	CO induces mitochondrial generation of O_2, which is converted by SOD to H_2O_2, and the subsequent Akt activation by H_2O_2 attenuates apoptosis during ischaemia–reperfusion	Kondo-Nakamura *et al.* (2010)
Neonatal cardiomyocytes and H9C2 cardiomyoblasts (CO and CORM-2)	Enhancement of neovascularization in the myocardium in the peri-infarct region and improvement of cardiac function	CO-mediated CXCL12 expression and Akt-dependent up-regulation	Lin *et al.* (2013)
Cardiac cells (pre-treatment with CORM-3)	Resistant to the damage caused by hypoxia-reoxygenation and oxidative stress	Cardioprotection was lost when CORM-3 was replaced by an inactive form (iCORM-3) that is incapable of liberating CO	Clark *et al.* (2003)
HUVEC (pre-treatment with CORM-2)	Preconditioning	Decrease of LPS-induced production of ROS and NO, up-regulation of HO-1, decrease of iNOS, inhibition of LPS-induced activation of NF-κB and down-regulation of expression of ICAM-1	Sun *et al.* (2008)
Rat isolated hearts (CO exposure)	Increased the baseline coronary flow and the contracture magnitude, improved contractile recovery and ventricular arrhythmia incidence, and increased the hyperemic coronary flow	CO-exposed hearts could be preconditioned in the same way as normal myocardium	Rochetaing *et al.* (2001)
Rat isolated hearts (PC with CORM-2)	Reduction of infarct size	Activation of the p38 MAPK β and PKC pathways before ischaemia and of PI3-kinase pathway during reperfusion	Soni *et al.* (2012)
Rat isolated hearts (PC with CORM-2)	Marked attenuation of infarct size	Activation of the K_{ATP} channels	Soni *et al.* (2010)
Mouse hearts (CORM-3)	Amelioration of LV dysfunction		Clark *et al.* (2009)
Isolated hearts (CORM-3)	Reduction in cardiac muscle damage and infarct size	Activation of mitoK_{ATP} channels	Clark *et al.* (2003)
Isolated hearts (CORM-2)	Reduction of infarct size	Activation of mitoK_{ATP} channels – NOS-cGMP and HO-1 pathways	Mei *et al.* (2007)
In vivo (mice) CORM-3 (24 h prior to coronary occlusion)	Reduction of infarct size	Delayed protection against myocardial infarction which is similar to that afforded by the late phase of PC	Stein *et al.*, 2005
In vivo (mice) CORM-3-pre-teatment	Reduction in markers of apoptosis after I/R injury	NF-κB, STAT1/3 and Nrf2 with a subsequent increase in cardioprotective and anti-apoptotic molecules	Stein *et al.* (2012)
In vivo (porcine) pre-treatment with low-dose CO	No protection as indicated by metabolic, energy-related and injury markers		Ahlström *et al.* (2011)

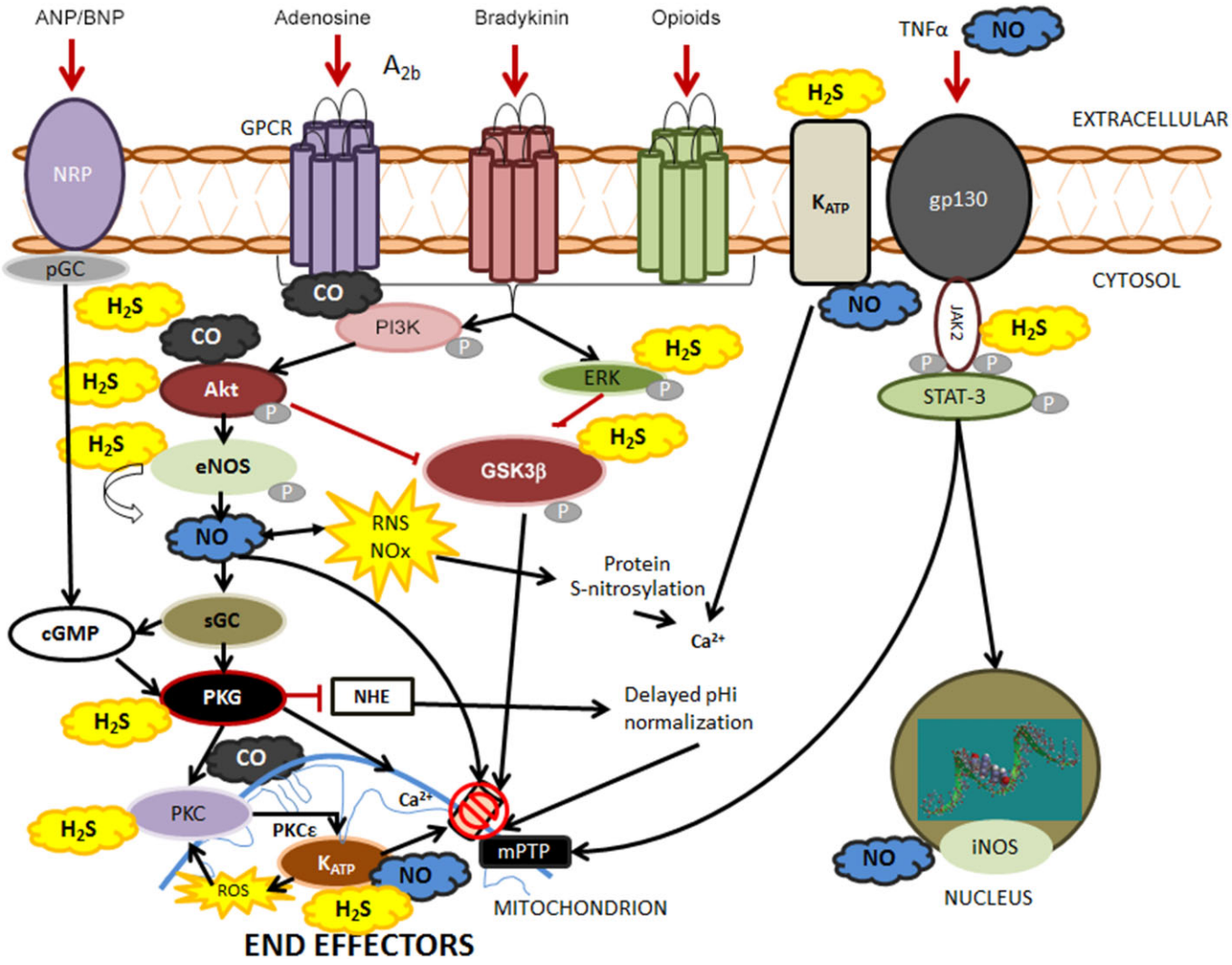

Figure 1

Schematic diagram showing major signalling cellular pathways of gasotransmitters NO (blue cloud), H_2S (yellow cloud) and CO (black cloud). ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; GSK3β, glycogen synthase kinase 3β; K_{ATP}, ATP-dependent potassium channel; mPTP mitochondrial permeability transition pore; sGC, soluble guanylyl cyclase.

rapamycin and probucol, have also been shown to induce HO-1 (Abraham and Kappas, 2008).

In conclusion, there is no large-scale clinical study providing solid evidence for the usefulness of therapy based on HO-1 in patients. As both CO and bilirubin play important roles in cardiovascular protection, the potential of these chemicals as clinical therapeutics as compared with HO-1 are still unclear and needs to be studied further.

Conclusions and future perspectives

In this review, we provided an overview of the recent advances in our knowledge on endogenously produced or pharmacologically administered NO, H_2S and CO in cardioprotection. Figure 1 summarizes the effects of the gasotransmitters on the major signalling pathways mediating cardioprotection. Table 4 summarizes the effect of gasotransmitters on I/R injury and cardioprotection by different conditioning strategies.

NO is an important component of several cardioprotective signalling cascades. Accordingly, NO donor molecules and compounds that activate NO-independent-cGMP-mediated signalling pathways are promising tools for cardioprotection in the clinical arena. For example, nitrite anion has emerged as a promising cardioprotective agent due to its ability to be reduced to NO in the ischaemic heart with an acidic environment. However, excess NO accumulation during ischaemia may contribute to reperfusion injury via nitrosative stress due to excess superoxide and the consequent peroxynitrite formation.

H_2S has recently come to the fore due to some promising data demonstrating that this gasotransmitter confers cardioprotection in a variety of settings. Both endogenously gener-

Table 4

Effect of gasotransmitters NO, H_2S and CO on major cardioprotective mechanisms

Gasotransmitter	I/R	Preconditioning	Postconditioning	Remote conditioning
NO endogenous	↓	↑	↑	Not clear
NO exogenous	↓	↑	Not clear	Not clear
H_2S endogenous	↓	↑	↑	Not studied
H_2S exogenous	↓	↑	Not clear	Not studied
CO endogenous	↓	↑	↑	Not studied
CO exogenous	↓	↑	Not studied	Not studied

ated H_2S and exogenously supplied H_2S reduce infarct size and improve heart function following I/R injury. Translational efforts aiming to move basic research findings into the clinical arena are underway with H_2S-releasing molecules.

CO is the least studied one of the three gasotransmitters in the context of ischaemic and pharmacological conditioning. Although HOs exert beneficial effects in I/R injury, the contribution of CO to this effect is not entirely clear. Nevertheless, some CORMs have been developed for cardioprotection; however, these molecules have not reached the clinical phase yet. Along with the excitement for the potential therapeutic use of CO comes the concern for its toxicity, as exposure to excess exogenous CO either acutely or chronically via air pollution has profound deleterious effects on cardiac function.

Findings from other research niches in the cardiovascular system have highlighted the importance of gasotransmitter crosstalk, suggesting that in I/R injury and cardioprotection, the role of gasotransmitters is worth investigating in an integrated way. Indeed, in angiogenesis and vasodilation, gasotransmitter pathways converge into common downstream effectors. Thus, successful therapeutic strategies might need to utilize more than one gasotransmitters or their pharmacological modulators.

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Author contributions

I. A. was responsible for conception and design, drafting of the manuscript and final approval of the manuscript submitted. E. K. I. was responsible for the critical revision of the manuscript and final approval of the manuscript submitted. T. R., R. S. and A. P. were responsible for the critical revision of the manuscript and final approval of the manuscript submitted. P. F. was responsible for the conception and design, drafting of the manuscript and final approval of the manuscript submitted.

Conflict of interest

None.

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