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Themed Section: Conditioning the Heart – Pathways to Translation

REVIEW

Remote conditioning the heart overview: translatability and mechanism

Michael Rahbek Schmidt[1], Andrew Redington[2] and Hans Erik Bøtker[1]

[1]*Department of Cardiology, Aarhus University Hospital Skejby, Aarhus N, Denmark, and*
[2]*Division of Cardiology, Hospital for Sick Children, Toronto, ON, Canada*

Correspondence
Hans Erik Bøtker, Department of Cardiology, Aarhus University Hospital Skejby, Brendstrupgaardsvej 100, DK-8200 Aarhus N, Denmark.
E-mail: heb@dadlnet.dk

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Conditioning the heart to resist predictable and unpredictable ischaemia–reperfusion (IR) injury is one of the fastest growing areas of bench to bedside research within cardiology. Basic science has provided important insights into signalling pathways and protective mechanisms in the heart, and a growing number of clinical studies have, with important exceptions, shown the potential applicability and beneficial effect of various mechanical conditioning strategies achieved by intermittent short-lasting-induced ischaemia of the heart itself or a remote tissue. Remote ischaemic conditioning (RIC) in particular has been utilized in a number of clinical settings with promising results. However, while many novel 'downstream' mechanisms of RIC have been discovered, translation to pharmacological conditioning has not yet been convincingly demonstrated in clinical studies. One explanation for this apparent failure may be that most pharmacological approaches mimic a single instrument in a complex orchestra activated by mechanical conditioning. Recent studies, however, provide important insights into upstream events occurring in RIC, which may allow for development of drugs activating more complex systems of biological organ protection. With this review, we will systematically examine the first generation of pharmacological cardioprotection studies and then provide a summary of the recent discoveries in basic science that could illuminate the path towards more advanced approaches in the next generation of pharmacological agents that may work by reproducing the diverse effects of RIC, thereby providing protection against IR injury.

LINKED ARTICLES

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

Abbreviations

Cx43, connexin 43; eNOS, endothelial nitric oxide synthase; GIK, glucose-insulin-potassium; GLP-1, glucagon-like peptide 1; IHD, ischaemic heart disease; IR, ischaemia–reperfusion; MACCE, major adverse cardiac and/or cerebrovascular event; MI, myocardial infarction; miR, microRNA; MPTP, mitochondrial permeability transition pore; PCI, percutaneous coronary intervention; RIC, remote ischaemic conditioning; STEMI, ST-elevation myocardial infarction

Tables of Links

TARGETS	
GPCRs[a]	**Enzymes**[d]
Adenosine A_1 receptors	Akt (protein kinase B)
Adenosine A_3 receptors	Dipeptidyl peptidase 4
CXCR4	eNOS, endothelial NO synthase
GLP-1 receptors	GSK3, glycogen synthase kinase 3β
Nuclear receptors[b]	JAK
Mineralocorticoid receptor	PI3K
Catalytic receptors[c]	PKC-δ
Fibroblast growth factor receptor 2	**Ion channels**[e]
Insulin-like growth factor-1 receptor (IGFR1)	Connexin (Cx) 43
TNF-α receptors	Na_+–H_+ exchanger (SLC9)

LIGANDS
Adenosine
Atorvastatin
CXCL12 (SDF1),
Cyclosporin A
Erythropoietin
Exenatide
GLP-1
Metoprolol
Nicorandil
NO, nitric oxide
Rapamycin (sirolimus)
Spironolactone

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

Introduction

From acute events such as stroke and acute myocardial infarction (MI) to predictable circumstances such as elective surgery and angioplasty, the injury caused by ischaemia and reperfusion is a leading cause of death and disability (Murray and Lopez, 1997; Wang *et al.*, 2012). Since 1990, more people have died from coronary heart disease than any other cause of death (Lloyd-Jones *et al.*, 2009; 2010). Ischaemia–reperfusion (IR) syndromes remain a major clinical challenge worldwide.

In acute coronary events, early and successful restoration of myocardial reperfusion following an ischaemic event is the most effective strategy to reduce final infarct size and improve clinical outcome, but reperfusion may induce further myocardial damage itself, so-called reperfusion injury (Murry *et al.*, 1986). Although the process of myocardial reperfusion continues to improve with more timely and effective coronary intervention and antiplatelet and antithrombotic agents for maintaining the patency of the infarct-related artery, the development of effective drugs to treat the detrimental effects of reperfusion injury itself has proven to be a challenge. Indeed, several pharmacological strategies showing convincing effects in animal models of IR injury have failed to translate to clinical benefit.

Consequently, as the first generation of pharmacological conditioning studies {including large clinical trials such as AMISTAD-II (adenosine), APEX-MI (pexelizumab) and CREATE-ECLA [glucose-insulin-potassium (GIK) infusion]} (see Table 1) failed to show convincing effects, mechanical ischaemic conditioning strategies have dominated the more recent clinical trials.

Since its conceptual demonstration in 1986, local ischaemic *pre*conditioning of the heart (and subsequently other organs) achieved by intermittent sub-lethal periods of ischaemia prior to a longer lasting ischaemic insult has evolved into the more clinically applicable methods of local ischaemic *post*conditioning and *remote* ischaemic conditioning (RIC) (see below), both of which have shown promising results in clinical trials (Staat *et al.*, 2005; Hoole *et al.*, 2009; Botker *et al.*, 2010; Lonborg *et al.*, 2010; Thielmann *et al.*, 2010; 2013; Davies *et al.*, 2013; Sloth *et al.*, 2014). The increasing insight into the mechanisms and pathways of ischaemic conditioning may pave the road for development of a new generation of cardioprotective drugs closely mimicking the powerful inherent protection afforded by ischaemic conditioning.

This review will focus upon novel advances in our understanding of the mechanisms involved in RIC and the scope for potential development of novel pharmacological approaches.

Remote ischaemic conditioning

'Remote ischaemic conditioning' (RIC), induced by repeated short-lasting ischaemia in a distant tissue – largely achieved by intermittent interruption of circulation in a limb – has recently emerged as a promising adjunctive therapy to avoid organ damage, thereby improving the outcomes of well-established therapies. From the site of the remote stimulus, through humoral (Shimizu *et al.*, 2009) and neuronal (Loukogeorgakis *et al.*, 2005; Lim *et al.*, 2010) pathways, RIC activates several protective mechanisms in the target organ similar to those activated by local preconditioning. Furthermore, RIC modifies the systemic inflammatory response (Konstantinov *et al.*, 2004; Shimizu *et al.*, 2010), prevents endothelial dysfunction (Kharbanda *et al.*, 2002) and platelet

Table 1

Pharmacological adjunctive conditioning therapy in myocardial infarction

	Intervention	N	Clinical scenario	Outcome
Adenosine				
Mahaffey *et al.* (1999) (AMISTAD)	Infusion of adenosine for 3 h	236	STEMI – thrombolysis	Reduction in infarct size
Kloner *et al.* (2006) (AMISTAD II)	Infusion of adenosine for 3 h	2118	STEMI – thrombolysis or primary PCI	No difference in death or HF
Atorvastatin				
Kim *et al.* (2010) (STATIN-STEMI)	Oral atorvastatin 80 mg before primary PCI	171	STEMI – primary PCI	No difference in death, revascularization or infarct size
Hahn *et al.* (2011b)	Oral atorvastatin 80 mg before primary PCI	173	STEMI – primary PCI	No difference in infarct size
Cyclosporin A				
Piot *et al.* (2008)	Cyclosporin infusion before primary PCI	58	STEMI – primary PCI	Reduction in infarct size
Erythropoietin				
Voors *et al.* (2010)	Single dose EPO	529	STEMI – primary PCI	No difference in LVEF or infarct size
Exenatide				
Lonborg *et al.* (2012b)	Infusion of exenatide for 6 h	107	STEMI – primary PCI	Reduction in infarct size
Glucose-insulin-potassium (GIK)				
Mehta *et al.* (2005) (CREATE-ECLA)	Infusion of GIK for 24 h	20 201	STEMI – thrombolysis	No difference in mortality
Selker *et al.* (2012) (IMMEDIATE)	Out-of-hospital infusion of GIK	357	STEMI – thrombolysis	Reduced mortality among patients with cardiac arrest
PKC-δ inhibitor				
Bates *et al.* (2008)	2 doses of KAI 9803	154	STEMI – primary PCI	No difference in infarct size

HF, heart failure; LVEF, left ventricular ejection fraction.

activation (Pedersen *et al.*, 2011) following IR injury. In experimental studies, RIC has been shown to afford protection against IR in the liver (Kanoria *et al.*, 2006; Lai *et al.*, 2006), lung (Harkin *et al.*, 2002; Jan *et al.*, 2011), kidney (Ali *et al.*, 2007), brain (Hahn *et al.*, 2011a), heart (Kharbanda *et al.*, 2002) and against cardiopulmonary bypass-induced neural, pulmonary and myocardial damage (Kharbanda *et al.*, 2006).

Direct translation of RIC into clinical use

In proof-of-principle randomized clinical trials based upon surrogate endpoints such as biomarkers and imaging, RIC has been shown to protect against IR injury in the heart (Cheung *et al.*, 2006; Hoole *et al.*, 2009; Botker *et al.*, 2010; Thielmann *et al.*, 2010), brain (Hougaard *et al.*, 2014), kidney (Lazaris *et al.*, 2009) and lung (Cheung *et al.*, 2006).

Three recent clinical follow-up studies indicate that RIC also confers prognostic benefit to the patient. An original study demonstrating reduced myocardial injury in patients treated with RIC prior to coronary artery by-pass surgery (Thielmann *et al.*, 2010) in a subsequent follow-up study showed that cardiac mortality and major adverse cardiac and/or cerebrovascular event (MACCE) were reduced among RIC-treated patients (Thielmann *et al.*, 2013). In a study investigating patients undergoing elective percutaneous coronary intervention (PCI), RIC not only reduced periprocedural myocardial injury (Hoole *et al.*, 2009) but also MACCE rate up to 6 years after the coronary intervention (Davies *et al.*, 2013). Finally, in a study utilizing RIC during ambulance transport to hospital in patients admitted with acute MI, RIC increased myocardial salvage (Botker *et al.*, 2010), which, up to 4 years after the infarction, translated into a 35% reduction in MACCE and a 52% reduction in all-cause mortality (Sloth *et al.*, 2014).

Although an increasing amount of evidence thus indicates that the cardioprotective effect of RIC can be translated into beneficial clinical effects, it is important to stress that properly sized studies with clinical endpoints, such as the ERICCA trial (ClinicalTrials.gov NCT01247545), RIP-Heart study (ClinicalTrials.gov NCT01067703) and the CONDI 2 trial (ClinicalTrials.gov NCT01857414), are needed.

Mechanisms of RIC

The organ-protective effects of RIC are at least partially mediated through the release of endogenous substances into the bloodstream, as plasma from RIC-treated animals is cardioprotective. Moreover, the same plasma can be dialysed and the dialysate applied to a naïve isolated heart to achieve

cardioprotection equal in strength to cardioprotection in hearts from RIC-treated animals (Shimizu *et al.*, 2009). Similarly, in a cardiac transplant model, RIC of a recipient animal reduces IR injury in the subsequently transplanted (denervated) donor heart, again suggesting the presence of a powerful humoral component to the RIC stimulus (Konstantinov *et al.*, 2005a). Although the exact nature of the circulating effector(s) is not yet clear, the signalling cascade induced by RIC has similarities with local ischaemic *pre*conditioning, there being a 'window' of cardioprotection lasting 2–6 h after the conditioning stimulus. Interestingly, RIC also induces a portfolio of myocardial gene expression responses associated with the stress-response and repair mechanisms (Konstantinov *et al.*, 2005b). Consequently, RIC induces later 'windows' of protection (a second window re-emerging at 12–24 h, and other chronic effects when RIC is delivered for days after an event) (Wei *et al.*, 2011), but their underlying biology is only partially understood and is beyond the scope of the current review.

As both local and remote ischaemic conditioning activate a multitude of pathways and mechanisms (Zhao *et al.*, 2003), eventually resulting in a complex and powerful organ protection, and experimental models rarely allow for investigation of more than one or two systems, a complete understanding of the underlying protective mechanisms is still lacking. Furthermore, a wide range of animal models, conditioning protocols and variable endpoints add to the puzzle. Nevertheless, a consensus on how to categorize the components of cardioprotection is emerging and a distinction between the three levels of signal transduction – triggers, intracellular mediators and effectors – has been suggested by Heusch *et al.* (Heusch *et al.*, 2008; Heusch, 2013) and Kharbanda *et al.* (2009).

Triggers are substances that are released as an immediate response to the conditioning stimulus. These include adenosine, bradykinin and opioids that activate sarcolemmal receptors thereby initiating the intracellular mediators, mainly consisting of protein kinases that finally act on the effectors, which include mitochondria, connexins and cytoskeletal elements. In line with this conceptual understanding, three individual intracellular pathways have been identified; the nitric oxide-dependent GPCR–endothelial nitric oxide synthase (eNOS)–PKG pathway; the reperfusion-injury salvage kinase pathway based upon PI3K-Akt and glycogen synthase kinase 3β (GSK3β; Hausenloy *et al.*, 2012b); and the survivor activating factor enhancement (Lecour, 2009; Tamareille *et al.*, 2011; Heusch *et al.*, 2012) signalling pathway involving the JAK-STAT system and TNF-α receptors (Heusch *et al.*, 2008; Heusch, 2013), all eventually acting on the mitochondria to modify their energetic and membrane integrity (Figure 1).

However appealing this schematic framework appears, we need to point out that it cannot be taken for a biological reality. Indeed, several recent studies indicate that the biological defence system activated by ischaemic conditioning may be far more complex in its nature. For example, the triggers mentioned earlier seem to act in concert with inhibition of platelet and neutrophil activation, and other anti-inflammatory and exosome-based microRNA signalling cascades (see below). Similarly, effector mechanisms may involve more complex long-term effects such as stimulating autophagy and modifying myocardial remodelling, later after the IR event.

First-generation pharmacological strategies and trials

The translation of mechanisms involved in mechanical conditioning into development of pharmacological agents has been attempted for decades.

The first clinical applications of pharmacological conditioning strategies reproduced a single step in cardioprotective signal transduction, mostly based upon findings concerning local ischaemic conditioning. Importantly, the majority of pharmacological cardioprotection trials are based upon experimental evidence of the effect of the drugs in question when administered *prior to* myocardial ischaemia, whereas the trials mostly tested their effect *during* evolving MI.

While many are negative, several of these trials have tested relevant putative agents and have provided important information and, hence, a condensed overview of the most prominent trials utilizing drugs sharing known or likely mechanistic properties with ischaemic conditioning will be provided. Several excellent and more comprehensive reviews covering pharmacological conditioning studies have been published recently (Gerczuk and Kloner, 2012; Hausenloy and Yellon, 2013; Heusch, 2013), and as the present paper primarily focuses upon the scope for new drug discovery, the summary below mainly serves as a preliminary to the discussion of potential future pharmacological conditioning.

Adenosine

Early discoveries of the importance of adenosine receptors in the upstream signalling of ischaemic conditioning led to intense investigation of the effect of adenosine itself or adenosine receptor agonists in various models and scenarios of IR injury. Several adenosine receptors are expressed in the myocardium and their individual role in the various forms of cardioprotection is not fully clarified, but stimulation of adenosine A_1 and A_3 receptors has repeatedly been shown to induce cardioprotection in experimental models (Toombs *et al.*, 1992; Zhao *et al.*, 1993). The protective effects appear to be mediated through PKC and ultimately achieved by inhibition of mitochondrial permeability transition pore (MPTP) formation (McIntosh and Lasley, 2012).

The AMISTAD-I trial (Mahaffey *et al.*, 1999) was one of the first clinical trials investigating pharmacological conditioning as an adjunct to acute myocardial reperfusion therapy in patients admitted with ST-elevation myocardial infarction (STEMI). This randomized placebo-controlled multi-centre trial involving 236 patients showed that adenosine infusion in patients treated with thrombolytic therapy for anterior wall STEMI reduced infarct size up to 50%. The following large-scale AMISTAD II (>2000 patients, Table 1) investigated the effect of adenosine infusion in patients undergoing thrombolysis or primary PCI for anterior STEMI (Ross *et al.*, 2005; Kloner *et al.*, 2006). No difference in the primary clinical endpoint (death or heart failure) was achieved by adenosine, although a *post hoc* analysis showed that among patients who received early (<3.17 h from symptom onset) reperfusion therapy, those that also received adenosine infusion had lower mortality and fewer events (in-hospital congestive heart failure or rehospitalization for congestive heart failure).

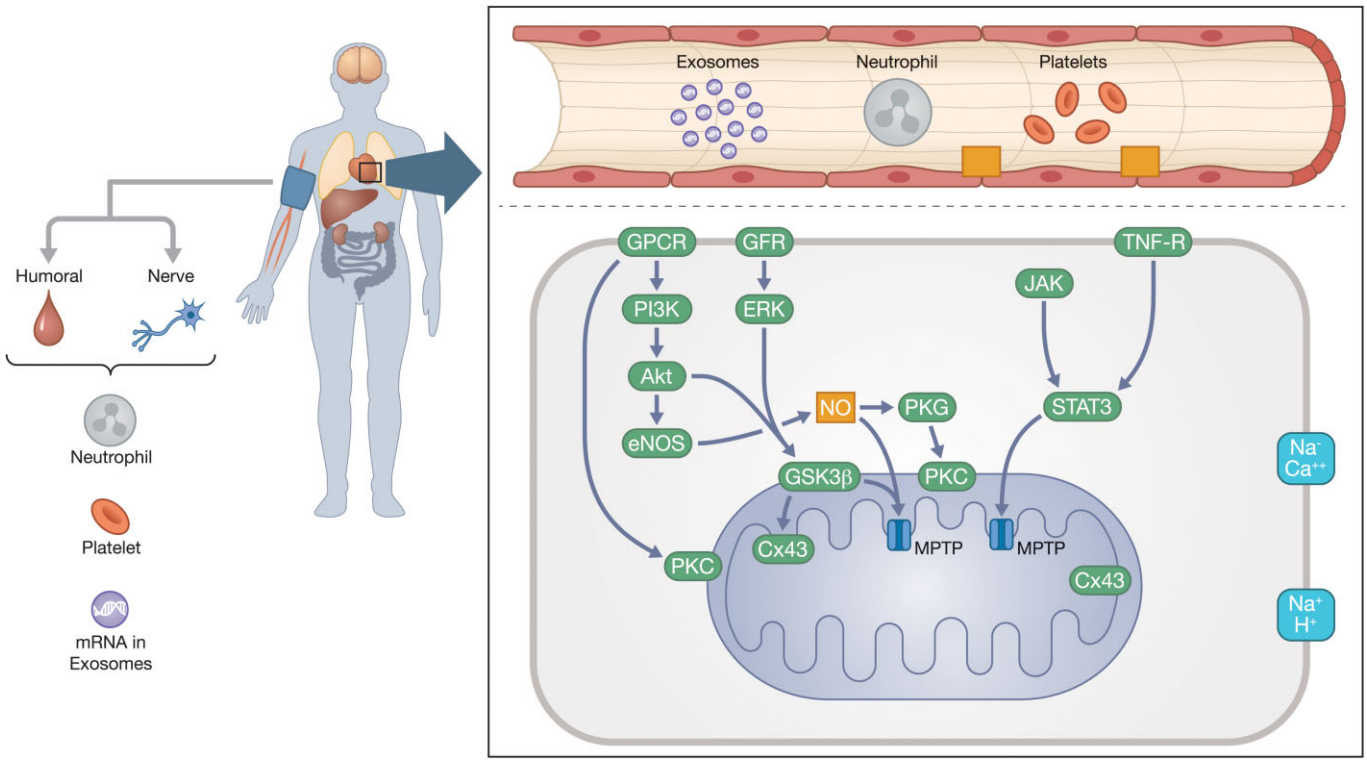

Figure 1

Simplified schematic presentation of the cytosolic signalling pathways that converge to prevent mitochondrial permeability transition pore (MPTP) opening in cardioprotection. eNOS/PGK: the nitric oxide-dependent GPCR–eNOS–PKG pathway; RISK: the reperfusion-injury salvage kinase pathway based upon PKB, PI3K-Akt and GSK3ß; and SAFE: the survivor activating factor enhancement signalling pathway involving the JAK-STAT system and TNF-α receptors. Proteins implicated in MPTP formation include the matrix cyclophilin D (CyD), the inner membrane adenine nucleotide translocase and the outer membrane voltage-dependent anion channel. Additional proteins such as the translocator protein 18 kDa (TSPO), located in the outer mitochondrial membrane, interact with proteins involved in MPTP formation. Under pathophysiological conditions, such as high Ca^{2+} concentration and increased oxidative stress, the complex forms an open pore between the inner and outer membranes that ultimately results in mitochondrial swelling, mitochondrial Ca^{2+} efflux and the release of apoptogenic proteins. Cyclosporin A targets matrix CyD, where Ca^{2+} overload triggers MPTP opening. TRO40303 binds to TSPO in the outer membrane. Other abbreviations: eNOS, endothelial nitric oxide synthase; GFR, growth factor receptors (insulin-like growth factor-1 and fibroblast growth factor-2); GSK3ß, glycogen synthase kinase 3ß; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; TNF-R, tumour necrosis factor receptor.

Intracoronary bolus administration immediately prior to reperfusion has not convincingly shown clinical effect, but timing, route and duration of adenosine administration seem crucial, and the optimal therapeutic protocol may not yet have been discovered. Such a protocol may include pretreatment with isoprenaline.

Beta blockers

Cardioselective beta blockers (β-adrenoceptor antagonists) are among the oldest and best-known pharmacological agents used in the treatment of acute and chronic ischaemic heart disease (IHD). Although their position in the modern multi-drug treatment era is frequently discussed, few dispute their beneficial effects in the treatment of acute MI and chronic IHD. Their mechanism of action in chronic IHD is complex and is beyond the scope of this paper, and the acute effects are incompletely understood.

Nevertheless, beta blockers have regained attention lately after the preclinical and clinical studies by Ibanez and colleagues have shown that administration of i.v. metoprolol immediately prior to reperfusion decreased infarct size and improved left ventricular function. In the recent METOCARD-CNIC trial conducted by the same group, i.v. metoprolol administered prior to primary PCI not only reduced myocardial injury but also improved long-term ventricular function and resulted in fewer admissions due to heart failure (Ibanez *et al.*, 2013). Whether these acute cardioprotective effects of beta blockers were achieved through activation of signalling pathways involved in ischaemic conditioning remains to be determined.

Mitochondria, cyclosporin A and the permeability transition pore

Mitochondria are generally considered among the most important final effectors in ischaemic conditioning, and in particular the MPTP has attracted much attention as it is closed during myocardial ischaemia but opens during reperfusion (Griffiths and Halestrap, 1995), which causes mitochondrial swelling, loss of function and, potentially, cellular necrosis. This opening of the MPTP can be modified by

ischaemic *post*conditioning as described previously or by pharmacological intervention with, for example, cyclosporin A (Hausenloy *et al.*, 2002; Waldmeier *et al.*, 2002).

Encouraging experimental studies supporting this hypothesis facilitated translation into clinical trials. Cyclosporin A has been shown to be a promising candidate for pharmacological conditioning agents in clinical studies, most conspicuously in the treatment of acute MI. Notably, Piot *et al.* in a study of 58 patients admitted with STEMI, randomized subjects to receive a bolus of cyclosporin A or normal saline immediately prior to reperfusion treatment with primary PCI. The authors found that cyclosporin A reduced the release of troponin I and improved left ventricular remodelling compared with controls (Piot *et al.*, 2008; Mewton *et al.*, 2010). Thorough reviews of the MPTP as a pharmacological target and of cyclosporin A as a cardioprotective agent are provided by Halestrap (2010) and Hausenloy *et al.* (2012a). The CIRCUS (NCT01502774) trial is currently investigating the clinical effects of cyclosporin A in patients admitted with STEMI.

Other pharmacological approaches to prevent or modify the MPTP opening by, for example, volatile anaesthetics (Piriou *et al.*, 2004) or metformin (Bhamra *et al.*, 2008) have shown cardioprotective capacity in experimental studies, but remain to be tested systematically in clinical trials. Similarly, other tactics to manipulate mitochondrial function, for example, through the mitochondrial respiration and electron transport chain may have potential pharmacological perspectives (see below). Most recently, the MITOCARE study investigating the effect of TRO40303, another drug targeting the MPTP, was launched and first results are expected later this year (MITOCARE Study Group, 2012).

The pH hypothesis

Rapid restoration of intracellular pH during reperfusion after the severe acidosis occurring during ischaemia is afforded by washout of lactate and activation of the Na^+–H^+ exchanger. The resulting rapid increase in pH is believed to generate detrimental effects, including opening of the MPTP and myocardial hypercontracture, eventually resulting in lethal myocardial reperfusion injury (the 'pH hypothesis'). Slowing down the pH restoration by infusion of an acidic buffer reduces IR injury, and a similar effect can be achieved by pharmacological inhibition of the Na^+–H^+ exchanger. It is likely that the cardioprotection associated with local postconditioning (stuttered reperfusion by intermittent coronary balloon occlusion), at least in part, is mediated via this effect (Lemasters *et al.*, 1996).

Interestingly, RIC also attenuates hypercontracture during early reperfusion in a porcine model (Schmidt *et al.*, 2007), indicating that RIC may also influence intracellular pH, although this finding may be indirect, via RIC's effect on the MPTP.

Pharmacological attempts to control or slow down the restoration of intracellular pH by inhibition of the Na^+–H^+ exchanger showed very promising cardioprotective effects in animal models. However, two large clinical trials (GUARDIAN and EXPEDITION) (Boyce *et al.*, 2003; Mentzer *et al.*, 2008) both exploiting the principle of inhibition of the Na^+–H^+ exchanger by cariporide failed to show clinical benefit of the treatment. Again, drug, timing and treatment protocol may not have been fully optimized to mimic the physiological concept of 'gentle reperfusion', and pharmacologically decelerated pH restoration may still find its way into clinical practice.

Insulin and glucagon-like peptide 1 (GLP-1) analogues

Metabolic support with glucose and insulin to alleviate substrate drought during ischaemia and reperfusion is a theoretically attractive principle that has been extensively investigated over several decades. Infusions of a combination of glucose and insulin and potassium salts (GIK) improved cardiac function, modified myocardial energetics and reduced infarct size in a wide range of experimental models of IR syndromes. Furthermore, recent animal studies show that metabolic support with glucose and insulin may be a prerequisite to achieve cardioprotection by RIC in the carbohydrate-dependent heart of the newborn (Schmidt *et al.*, 2014).

Clinically, GIK infusion has been shown to improve myocardial recovery after revascularization surgery or angioplasty in both diabetic and non-diabetic populations (Majid *et al.*, 1972; Coleman *et al.*, 1989; Gradinac *et al.*, 1989; Depre *et al.*, 1999; Kjellman *et al.*, 2000; Lazar *et al.*, 2000), although the clinical relevance of the results have been debated (Bruemmer-Smith *et al.*, 2002; Smith *et al.*, 2002; Castro *et al.*, 2003). Similarly, metabolic support based upon amino acid-enriched cardioplegic solutions was shown to afford cardioprotection in some early studies of MI (Bull *et al.*, 1984; Robertson *et al.*, 1984; Rosenkranz *et al.*, 1984; 1986; Julia *et al.*, 1991; Kofsky *et al.*, 1991), but later studies have not confirmed this effect (Uyar *et al.*, 2005). GIK infusion during transport to primary PCI, however, may reduce IR injury (Selker *et al.*, 2012).

Equally intriguing and potentially more easily clinically applicable is to use the gut incretin GLP-1, which, apart from its direct metabolic effects, activates anti-apoptotic signalling pathways such as PI3K and MAPK. GLP-1 receptors are present on cardiomyocytes, and GLP-1 and its analogues have been shown to protect against myocardial reperfusion injury (Bose *et al.*, 2005; Zhao *et al.*, 2006; Sonne *et al.*, 2008). Among several GLP-1 analogues, exenatide has repeatedly been shown to provide cardioprotection in animal models (Sonne *et al.*, 2008) and was recently shown to reduce myocardial injury in patients admitted for primary PCI when administered prior to reperfusion (Lonborg *et al.*, 2012a,b).

Exenatide represents a modern biomimetic drug that exerts complex biological actions by activating upstream events in several physiological signalling cascades. While the mechanisms behind the cardioprotective effect of exenatide may not yet be fully elucidated, they are believed to include the stimulation of glucose over fatty acid metabolism, resulting in more oxygen-efficient ATP production and activation of the PI3K/Akt system also involved in ischaemic conditioning.

Dipeptidyl-peptidase 4 inhibitors seem to have similar cardioprotective effects, at least in diabetic animals (Huisamen *et al.*, 2011), and may prove to be of clinical importance in clinical cardiovascular disease (Hausenloy *et al.*, 2013).

Connexin 43 (Cx43)

Connexins are transmembrane proteins that assemble into hexameric structures (hemichannels), which interconnect cells by the formation of gap junctions by docking of two hemichannels (Boengler *et al.*, 2006). Connexin 43 (Cx43) is the predominant connexin isoform in the cardiomyocyte membrane and is also present in the inner cardiomyocyte mitochondrial membrane. During later phases of ischaemia, Cx43 levels are decreased, which leads to lowered gap junction communication. In contrast, Cx43 levels are increased by ischaemic preconditioning (Boengler *et al.*, 2005), and reduction of Cx43 abolishes the cardioprotection by ischaemic preconditioning (Boengler *et al.*, 2007) but not postconditioning (Schulz *et al.*, 2007). Mitochondrial Cx43 is targeted by several protein kinases, including GSK3, which links Cx43 to one of the down-stream signalling pathways of ischaemic conditioning (Boengler *et al.*, 2007; 2012).

Apart from its anti-arrhythmic effect, metoprolol seems to increase Cx43 protein levels, independent of protein kinases (Salameh *et al.*, 2010), which might indicate that metoprolol stabilizes Cx43 gap junctions or prevents Cx43 degradation, hence providing a mechanism for the benefits seen in the clinical trial (METOCARD-CNIC) of the acute effects of metoprolol in evolving MI.

Rotigaptide (ZP123) is one of the best-known modulators of Cx43 and has been shown in several experimental studies to protect against IR injury (Haugan *et al.*, 2006; Hennan *et al.*, 2006), although its mechanism of action is still not fully understood. While rotigaptide seems to prevent the ischaemia-related down-regulation/internalization of gap junctions, it seems to be its effect on the mitochondrial Cx43 that results in cardioprotection.

Clinically, limited data exist with rotigaptide and other connexin modulators. Preliminary data suggest a good clinical safety profile of rotigaptide (Kjolbye *et al.*, 2007), but no publications showing cardioprotective benefit in patients were found at the time of writing. Other modulators of Cx43 showing cardioprotective effects in experimental studies, such as GAP-134 and ZP-1609 (danegaptide), are currently under consideration for clinical trials.

Other (failed) pharmacological strategies

In smaller clinical studies, pretreatment with HMG-CoA inhibitors (statins) prior to revascularization reduced myocardial injury (Pasceri *et al.*, 2004), but later larger studies failed to show any clinically relevant effect of high dose atorvastatin prior to primary PCI (Kim *et al.*, 2010; Hahn *et al.*, 2011b; Post *et al.*, 2012). Similarly, the anti-inflammatory drugs pexelizumab (Armstrong and Granger, 2007), erythropoietin (Voors *et al.*, 2010), opioids and nicorandil (Kitakaze *et al.*, 2007), despite promising preclinical data, all failed to afford clinically relevant cardioprotection in larger clinical trials.

An example of direct intervention on a pathway involved in ischaemic conditioning, the PKC-δ inhibitor delcasertib also failed to reduce myocardial injury in patients admitted with STEMI for primary PCI (Lincoff *et al.*, 2014), despite PKC being one of the most intensively studied signalling steps in cardioprotection with several preclinical studies showing cardioprotection by pharmacological control of the PKC activity (Budas *et al.*, 2007). Most recently, a clinical trial (NIAMI) investigating the effect of i.v. sodium nitrite prior to reperfusion in STEMI patients admitted for primary PCI was negative (Siddiqi *et al.*, 2014). However, this was immediately followed by a study by Rassaf *et al.*, showing that circulating nitrite derived from shear stress-dependent stimulation of eNOS at the remote site of RIC contributes to cardioprotection during IR injury, and again timing, dose and delivery site seem crucial (Rassaf *et al.*, 2014a,b).

Translating RIC into new pharmacological strategies

The search for new adjunctive pharmacological therapies of myocardial IR injury is probably among the most active areas of research today. The repeatedly convincing findings of cardioprotective effects of various forms of ischaemic conditioning therapies in relation to both elective and acute clinical procedures have strongly encouraged the pursuit of drugs that reproduce mechanical conditioning. Below, we will discuss how the most recent and exciting insights into the mechanisms of RIC may contribute to the development of new drugs.

Mimicking triggers and mediators of RIC

Intuitively, the higher up the signalling cascade of ischaemic conditioning a pharmacological agent acts, the closer it will reproduce the complexity of the endogenous signalling system. Consequently, the understanding of what sparks the initial events in, for example, RIC is fundamental in the search for more advanced biomimetic drugs. The signal transduction cascade in RIC begins with neural stimulation followed by release of cardioprotective substances. While the cardioprotective effect of RIC is reliant on intact neural pathways (Steensrud *et al.*, 2010; Donato *et al.*, 2013), it appears possible to mimic the neuronal trigger by acupuncture (Gao *et al.*, 2006; Redington *et al.*, 2013). Furthermore, nociceptor stimulation by topical application of the irritant capsaicin also induces cardioprotection mediated by circulating factors (Jones *et al.*, 2009; Redington *et al.*, 2012). Interestingly, adenosine and NO may also play a role in upstream signalling of RIC as intra-arterial adenosine administration evokes cardioprotection (Liem *et al.*, 2002; Dong *et al.*, 2004) by circulating humoral factors, and this effect is abolished by nerve transection (Steensrud *et al.*, 2010). Although these findings are highly interesting, a larger pharmacological potential may lie in discovering the nature of the cardioprotective circulating humoral factor(s).

The elusive factor X: the Higgs boson of RIC?

Cardioprotective plasma from animals and humans exposed to RIC can be dialysed through a 12 kDa standard membrane, and the dialysate will subsequently protect naive hearts against IR injury (Shimizu *et al.*, 2009; Lim *et al.*, 2010; Jensen *et al.*, 2012; Michelsen *et al.*, 2012). The dialysate can even be frozen or its contents freeze-dried and remain cardioprotective. Furthermore, the eluate of a C18 column, over which cardioprotective dialysate has been passed, is also cardioprotective. Although poignantly termed 'Factor X', the small, hydrophobic, cardioprotective content of the dialysate most likely consists of several substances. Nevertheless, it is tempt-

ing to search for the trigger molecules in the dialysate as their identification could potentially lead to the development of pharmacological substitutes.

Recently, several studies have suggested putative candidates for factor(s) X. In a proteomic study, Hepponstall *et al*. identified a number of proteins (including α1-antitrypsin, apolipoproteins and haptoglobin) that were up-regulated in response to RIC (Hepponstall *et al*., 2012) but also observed a significant down-regulation of other (predominantly inflammatory) proteins, in accordance with a previous genomic study (Konstantinov *et al*., 2004). More direct evidence for a factor X comes from Davidson *et al*. They showed that the chemokine CXCL12 (SDF1) and its receptor CXCR4 were crucial to the cardioprotection afforded by RIC, as RIC increased levels of CXCL12, cardioprotection could be induced by administration of CXCL12, and the effect of RIC could be blocked by a CXCR4 antagonist, although only partly, suggesting that other factors may be at play, or that release of this chemokine was an epiphenomenon downstream of another effector.

MicroRNA and exosomes

RNA is usually rapidly degraded in the blood and mainly serves as an intracellular mediator of protein transcription, but recently small non-coding RNAs that circulate in a stable form in blood and regulate gene expression post-transcriptionally have been identified. Such microRNAs (miRNAs or miRs) seem to act as both mediators of and protection against disease, and furthermore may serve as biomarkers of activation of protective conditions or other biological reactions to external stimuli.

Recent studies suggest that miRNAs are also involved in the signalling cascade of IR injury and ischaemic conditioning. Indeed, miRs have been shown to be effector molecules in ischaemic events (Li *et al*., 2014; Varga *et al*., 2014), and in one study, limited to analysis of two miRs (miR-1 and miR-21), there was differential expression depending upon the type of stimulus (local vs. remote) (Kukreja *et al*., 2011).

miRs appear to be intimately involved in the RIC stimulus. In a recent study, myocardial miR-144 levels were shown to be markedly reduced by IR injury alone (Li *et al*., 2014). The RIC stimulus increased plasma levels of miR-144 in mice and human volunteers, and RIC markedly attenuated the reduction in myocardial miR-144 levels in mice subjected to IR injury. Furthermore, i.v. administration of the miR-144 antagonist (antagomiR-144) abolished the cardioprotection afforded by RIC. Perhaps even more exciting was the finding that i.v. administration of miR-144 not only induced early cardioprotection (<60 min) associated with induced autophagy and increased phospho-Akt, phospho-GSK and phospho-p44/42 MAPK signal but also a delayed window of protection at 24 h and 3 days after IR injury, including down-regulation of the mammalian target of rapamycin (mTor). mTor is a key negative regulator of autophagy and a known target gene of miR-144. Pharmacological stimulation of mTor using rapamycin has been shown to modify post-MI remodelling (and subsequent development of heart failure) in a mouse model, much as has been shown for RIC when administered daily for 28 days after MI in rats. Although not yet studied directly, treatment with miR-144 may therefore provide early, and later, cardioprotection against the acute and chronic effects of myocardial IR injury in patients.

There is growing evidence for the existence of small vesicles, termed exosomes, that act as key components in the transport of miRNAs or their precursors. Although direct evidence for exosome trafficking, containing a nucleic acid signalling cargo, to ischaemic sites and the fate of the exosomes is lacking, the discovery of exosomes has presented a fascinating signalling system in the body. Indeed, miRNAs have been shown to be effector molecules in ischaemic events (Kukreja *et al*., 2011; Bhalala *et al*., 2013), with compelling evidence for a prominent role of exosomes in distant cell–cell transport. Exosomes enriched with miR-22 and miR-451 from anoxic cultured muscle stem cells (Feng *et al*., 2014) and cardiomyocyte progenitor cells (Chen *et al*., 2013) reduced cardiac damage in mouse myocardial ischaemia models. In relation to ischaemic conditioning, Ferdinandy's group recently demonstrated increased exosome release in the coronary effluent of locally preconditioned hearts, and that those exosomes were cardioprotective (Giricz *et al*., 2014). Furthermore, in this study of miR-144 biology in relation to RIC, the double-stranded hairpin precursor of miR-144 was increased over fourfold in the exosomes of animals subjected to RIC, using transient lower limb ischaemia.

Based upon conditioning-specific miRNA signatures, it may be possible to develop an exosome-based delivery system with a multifaceted RNAi payload capable of either sequestering using miRNA sponges or antagomiRs targeted at detrimental miRNAs associated with disease progression or introduction of beneficial miRNAs to alleviate disease burden, ultimately leading to the utilization of exosomes as nanocarriers of nucleic acid-based therapeutics in personalized treatment.

Mimicking effectors of ischaemic conditioning within the cell

The intensive studies of the effector mechanisms of ischaemic conditioning have identified a number of potential targets for pharmacological intervention. Of these, the MPTP and other mitochondrial signalling systems appear to be the most promising candidates for pharmacological intervention.

DJ-1

DJ-1 is a widely expressed and conserved mitochondrial protein that has been implicated in numerous pathologies, most notably in neurodegeneration where mutations in DJ-1 ultimately result in an early onset, familial form of Parkinson's disease (Bonifati *et al*., 2003). In relation to IR injury, DJ-1 has interesting properties, including oxidative stress sensing and reactive oxygen species scavenging. In a recent study by Dongworth *et al*. in a murine model of IR injury, overexpression of DJ-1 delayed MPTP opening and reduced cell death, whereas DJ-1 deficiency increased cell death and mitochondrial fragmentation (Dongworth *et al*., 2014). DJ-1 can be up-regulated by sodium 4-phenylbutyrate and may therefore represent a new therapeutic target.

Mineralocorticoid receptor antagonists

It is well established that the mineralocorticoid receptor antagonist spironolactone improves prognosis in patients

with chronic heart failure (Pitt *et al.*, 1999). Paradoxically, high plasma levels of aldosterone are associated with increased mortality in acute cardiovascular disease (Beygui *et al.*, 2006).

Nonetheless, administration of mineralocorticoid receptor antagonists before ischaemia (Chai *et al.*, 2005; 2006) or at time of reperfusion (Schmidt *et al.*, 2010) reduces myocardial IR injury in animal models, and this effect appears to be independent of aldosterone itself and shares mechanisms with ischaemic conditioning. For a thorough review, please see van den Berg *et al.* (2014).

Currently, two large trials [ALBATROSS (NCT01059136) and REMINDER (NCT01176968)] are investigating the effects of mineralocorticoid receptor antagonists in patients admitted with acute coronary syndrome.

The heart outside the cardiomyocyte

RIC attenuates IR injury-related endothelial dysfunction through mechanisms that are generally believed to be NO dependent (Heusch *et al.*, 2008) and the well-described cardioprotective effects of the NO donor S-nitroso-N-acetylpenicillamine may also be exerted through preservation of endothelial function. Similarly, microvascular obstruction is also important in relation to myocardial IR injury, and new imaging techniques allow for visualization of capillary function (Ostergaard *et al.*, 2014) and better understanding of microvascular dysfunction. Hence, vascular dysfunction after ischaemia may prove to be another important part of IR injury that can be targeted by pharmacological intervention, albeit beyond the scope of the current article.

Although IR injury is partly caused by an inflammatory response to ischaemia, pharmacological anti-inflammatory agents such as pexelizumab (antibody to the C5 component of the complement system) have not shown clinical effect in larger trials (Armstrong and Granger, 2007). Another anti-inflammatory agent, the naturally occurring fibrin-derived peptide FX06, reduced infarct size in patients treated with primary PCI for STEMI (Atar *et al.*, 2009) in a proof-of-concept study but has not yet been tested in a large-scale trial.

Summary

The increasing insight into the mechanisms behind the cardioprotective effects of RIC has uncovered several targets for pharmacological intervention that potentially may partly reproduce the effects of mechanical conditioning. While the first generation of drugs used for adjunctive therapy against IR injury has been disappointing, newer trials investigating more advanced treatment strategies with GLP-1 analogues and cyclosporin A are more promising.

Importantly, the recent discoveries of microRNA signalling and exosome-based transport may open for a completely new field of pharmacological cardioprotection, potentially closely reproducing the biological effects of RIC.

As RIC induces a wide range of systemic effects including multi-organ protection, anti-inflammatory, reduced platelet activation and increased exercise performance, perhaps indicating that this inherent protection system in mammalian species is a fundamental and complex part of the biological response to stress, it may yet prove too complex to be fully reproduced by a single pharmacological intervention. Future work should consider combination therapies or 'up-stream' intervention, which may mimic the signalling systems sufficiently to achieve clinically relevant organ protection comparable to the effects of remote and local ischaemic conditioning.

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Conflict of interest

M. R. S., A. R. and H. E. B. are shareholders of CellAegis Inc.

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