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**POSTCONDITIONING SIGNALLING IN THE HEART:**

**MECHANISMS AND TRANSLATABILITY**

*Abbreviated title: Myocardial postconditioning*

**Justin S. Bice & Gary F. Baxter**

School of Pharmacy and Pharmaceutical Sciences, Cardiff University, UK

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Address for correspondence

Professor Gary F. Baxter Ph.D., D.Sc.  
School of Pharmacy and Pharmaceutical Sciences  
Cardiff University  
King Edward VII Avenue  
Cardiff CF10 3NB  
United Kingdom

Telephone +44 (0)29 2087 6309  
Fax +44 (0)29 2087 4149  
Email [baxtergf@cardiff.ac.uk](mailto:baxtergf@cardiff.ac.uk)

## Summary

The protective effect of ischaemic postconditioning (short cycles of reperfusion and reocclusion of a previously occluded vessel) was identified over a decade ago commanding intense interest as an approach for modifying reperfusion injury which contributes to infarct size in acute myocardial infarction. Elucidation of the major mechanisms of postconditioning has identified potential pharmacological targets for limitation of reperfusion injury. These include ligands for membrane-associated receptors, activators of phosphokinase survival signalling pathways and inhibitors of the mitochondrial permeability transition pore. In experimental models, numerous agents that target these mechanisms have shown promise as postconditioning mimetics. Nevertheless, clinical studies of ischaemic postconditioning and pharmacological postconditioning mimetics have been equivocal. The majority of experimental research is conducted in animal models which do not fully portray the complexity of risk factors and comorbidities with which patients present and which we now know modify the signalling pathways recruited in postconditioning. Cohort size and power, patient selection and deficiencies in clinical infarct size estimation may all represent major obstacles to assessing the therapeutic efficacy of postconditioning. Furthermore, chronic treatment of these patients with drugs like ACE inhibitors, statins, nitrates etc. may modify signalling, inhibiting the protective effect of postconditioning mimetics, or conversely, induce a maximally protected state wherein no further benefit can be demonstrated. Arguably, successful translation of postconditioning can not occur until *all* of these issues are addressed i.e. experimental investigation requires more complex models that better reflect the clinical setting, while clinical investigation requires bigger trials with appropriate patient selection and standardisation of clinical infarct size measurements.

**Keywords:** Infarction, postconditioning, reperfusion injury, myocardium, ischaemia, PCI, cardioprotection

## **Abbreviations**

5-HD: 5-hydroxydecanoate  
ACE: angiotensin converting enzyme  
ACS: acute coronary syndrome  
Akt: protein kinase B  
AMI: acute myocardial infarction  
ANP: atrial natriuretic peptide  
BNP: B-type natriuretic peptide  
cGMP: 3',5'-cyclic guanosine monophosphate  
CK: creatine kinase  
CK-MB: creatine kinase assay  
CMR: cardio magnetic resonance  
CYP-D: cyclophilin-D  
Cys-A: cyclosporine-A  
eNOS: endothelial nitric oxide synthase  
EPO: erythropoietin  
ERK: extracellular regulated kinase  
GSK-3 $\beta$ : glycogen synthase-3 $\beta$   
MK<sub>ATP</sub>: ATP sensitive mitochondrial potassium channel  
MPTP: mitochondrial permeability transition pore  
NO: nitric oxide  
NPR: natriuretic peptide receptor  
PCI: percutaneous coronary intervention  
pGC: particulate guanylyl cyclase  
PI3K: phosphatidyl inositol 3 kinase  
PKC: protein kinase C  
PKG: protein kinase G  
PLB: phospholamban  
RISK: reperfusion injury salvage kinase  
ROCK: Rho-associated protein kinase  
ROS: reactive oxygen species  
SAFE: survivor activating factor enhancement  
SERCA: sarcoplasmic/endoplasmic reticulum calcium ATPase  
sGC: soluble guanylyl cyclase  
STAT3: signal transducer and activator of transcription 3  
STEMI: ST-elevated myocardial infarction  
TNF: tumour necrosis factor

## ***Development of the postconditioning paradigm for cardioprotection***

Death due to acute myocardial infarction (AMI) has declined steadily in the economically developed countries during the last 50 years. Since the 1980s, the development of reperfusion therapies as the “standard of care” for AMI has contributed markedly to the decline in early mortality. However, while case fatality rate has declined, there is evidence of an increasing incidence of chronic heart failure in AMI survivors. It is likely that infarct size is a major determinant of myocardial remodelling processes that predispose to subsequent heart failure development. Thus, prompt reperfusion is necessary and effective to limit the development of ischaemic necrosis during AMI but it seems plausible that further limitation of infarction is desirable to reduce long term morbidity and mortality due to heart failure. The identification of potential adjunctive infarct-limiting treatments has been a goal of experimental cardioprotection research for several decades. A vast array of pharmacological and other interventions have been described. A review of all of these is beyond the scope of this paper but we wish to highlight here three pivotal conceptual developments that emerged over several decades and converged to provide a new cardioprotection paradigm around 2005.

1. A mechanism of tissue injury specifically associated with reperfusion, termed “lethal reperfusion injury”, had been proposed as long ago as the late 1970s (Ashraf *et al.*, 1978; Hearse *et al.*, 1978). This concept implied the rapid irreversible injury, or accelerated death, of cells still viable at the end of an ischaemic period as a result of the sudden reintroduction of oxygen to ischaemic tissue. This reperfusion-associated cell death would be expected to contribute to final infarct size in reperfused AMI. For two decades, the concept of lethal reperfusion injury proved to be controversial. The proposed molecular and cellular mechanisms of lethality were diverse and poorly

defined. Most importantly, experimental pharmacological interventions specifically targeted at reperfusion were not consistent in their infarct-limiting ability. However, from the mid-1990s, evidence was accruing that apoptotic signals are activated during early reperfusion and that reperfused myocardium displays hallmarks of apoptosis. Although the quantitative contribution of apoptosis to infarct size is likely to be small, experimental activation of anti-apoptotic survival signals and inhibition of caspases, were found to limit infarct size in experimental models. This work led to the development by Yellon and colleagues of a general hypothesis that attenuation of lethal reperfusion injury and limitation of infarct size could be induced by activating anti-apoptotic survival signals termed “reperfusion injury salvage kinases” (RISK pathway) (Hausenloy *et al.*, 2004).

2. In 1986, Murry *et al.* made the experimental observation that brief periods of ischaemia preceding AMI led to an acute adaptation of myocardium that limited infarct size (Murry *et al.*, 1986). This phenomenon was termed ischaemic preconditioning. Intensive research throughout the 1990s revealed that ischaemic preconditioning is associated with the recruitment of a number of autacoid-stimulated signal transduction mechanisms which enhance the tolerance of myocardium to ischaemia-reperfusion insult, and thereby limit infarct size. The first autacoid to be identified in relation to the preconditioning mechanism was adenosine. This factor had been investigated extensively prior to the discovery of preconditioning in relation to nucleotide metabolism in ischaemia (Berne *et al.*, 1974; Berne, 1980). Indeed, ATP catabolism had been an area of active investigation in the laboratory of Reimer and Jennings for many years (Reimer *et al.*, 1986) and led directly to the experimental protocol that identified preconditioning (Murry *et al.*, 1986). Subsequently through the 1990s several other autacoid factors and numerous intracellular signal transduction mechanisms

were identified, all presumed only to be effective if activated prior to the onset of AMI. While preconditioning mechanisms induce a marked and very reproducible infarct-limiting effect, the clinical utility of therapies based on these mechanisms is extremely limited since pre-ischaemic treatment is implausible for the majority of AMI patients in whom coronary thrombosis is sudden and unheralded.

3. In 2003, Vinten-Johansen and colleagues reported the observation that intermittent repetitive re-occlusion of the infarct-related coronary artery during the early moments of reperfusion in an experimental model of AMI was able to limit infarct size as effectively as ischaemic preconditioning (Zhao *et al.*, 2003). This reperfusion-specific intervention is termed ischaemic postconditioning. Subsequent early research on postconditioning was remarkable for several reasons. First, postconditioning confirmed that lethal reperfusion injury contributes significantly to final infarct size. Second, both ischaemic preconditioning and ischaemic postconditioning were shown to involve activation at reperfusion of the RISK pathway identified a few years previously (Kin *et al.*, 2004). Clearly, the temporal characteristics of postconditioning highlight the relative importance of reperfusion injury in AMI, but has no effect on ischaemic injury even though ischaemia is the *sine qua non* of AMI.

Introduction of the postconditioning paradigm for cardioprotection has attracted huge interest in the possibility of therapeutic intervention at reperfusion to limit the injurious combined effect of ischaemia and reperfusion. In this respect, intervention at reperfusion with conditioning protocols or with pharmacological agents that recapitulate conditioning mechanisms can truly be said to represent a paradigm shift in the field.



## ***Characteristics of postconditioning***

Interventions applied in the early reperfusion period to augment tissue salvage, beyond that achieved by reperfusion alone, are now often described as postconditioning treatments. Such interventions may take several forms and it is important to distinguish between them. Here we provide a brief overview of these interventions and their major characteristics: for further discussion the reader is referred to more detailed reviews elsewhere (Burley *et al.*, 2009; Ovize *et al.*, 2010; Shi *et al.*, 2012; Hausenloy, 2013).

### ***Myocardial ischaemic postconditioning***

Postconditioning to limit infarct size was first formally described and characterised in the open-chest dog by Zhao *et al.* (2003). This form of postconditioning is referred to as myocardial ischaemic postconditioning, classical postconditioning, or mechanical postconditioning. It has been described in several other experimental species (mouse, rat, rabbit and pig) *in vivo* (Yang *et al.*, 2004; Schwartz *et al.*, 2006; Tang *et al.*, 2006; Gomez *et al.*, 2008); in isolated rodent heart preparations (Tsang *et al.*, 2004; Heusch *et al.*, 2006); in humans and in isolated human myocardium (Sivaraman *et al.*, 2007). The major characteristic of the intervention is that brief (typically 10-30 second), repetitive periods (3-10 cycles) of ischaemia, interspersed with brief (10-30 second) periods of reperfusion, are achieved by physical occlusion and reperfusion of the infarct-related coronary artery immediately following the index ischaemic event (see Figure 1). Most studies suggest that the timing of the intervention is critical to the outcome in reducing infarct size. A delay of more than one minute in instituting the first re-occlusion of the coronary artery was associated with a loss of protection (Skyschally

*et al.*, 2009). This concurs with the prevailing view that lethal reperfusion injury, associated with opening of the mitochondrial permeability transition pore (MPTP), occurs within the first few minutes of reperfusion (Griffiths *et al.*, 1995; Di Lisa *et al.*, 2001). However, limited evidence in mouse heart suggests that myocardial ischaemic postconditioning can limit infarct size if instituted even 30 minutes after reperfusion (Roubille *et al.*, 2011). This effect has been termed “delayed” ischaemic postconditioning. Whether it is a phenomenon generalizable to other species, including humans, is not clear. However, it has been suggested that the observation supports the concept of a gradually evolving “wavefront of reperfusion injury”, susceptible to later intervention.

Although ischaemic postconditioning has been reported in every animal species examined, considerable variation in the extent of infarct limitation is seen between species and laboratories. Murine models of postconditioning typically display 30% relative reduction in infarct size whereas in larger models such as the rabbit and canine infarct size limitation is around 50% (Vinten-Johansen *et al.*, 2011). Considerable variation is found in the multitude of postconditioning protocols used and in the duration of index ischaemia employed in these experimental models. Some data suggest that the threshold for ischaemic postconditioning rises as index ischaemic duration increases. There have been some reports of the failure of ischaemic postconditioning to limit infarct size (Schwartz *et al.*, 2006; Dow *et al.*, 2007; Hale *et al.*, 2008). Typically those studies which failed to show infarct limitation following postconditioning used briefer index ischaemia, and for larger animals, shorter postconditioning cycles. It is clear that there is not one protocol that suits all models and differences in protocols may account for the varying degrees of protection.

In addition to limiting infarct size, ischaemic postconditioning has been reported to limit the severity of other deleterious consequences of reperfusion. These include the development of arrhythmias in the rat heart (Dow *et al.*, 2008), cardiomyocyte apoptosis and the extent of vascular injury (Schwartz *et al.*, 2012).

### *Remote ischaemic postconditioning*

Numerous experimental and clinical observations suggest that intermittent ischaemia at the onset of myocardial reperfusion of tissues and organs remote from the heart can limit myocardial infarct size (see Figure 1). This phenomenon, called remote ischaemic postconditioning (or inter-organ postconditioning), is the subject of a comprehensive review elsewhere in this issue (Schmidt *et al.* this issue). The most frequently applied remote ischaemic postconditioning intervention, in both experimental and clinical models, is intermittent limb ischaemia performed at the onset of myocardial reperfusion (Kharbanda *et al.*, 2001; Loukogeorgakis *et al.*, 2006). The potential utility of such a simple intervention (e.g. repeated inflation of a blood pressure cuff) has attracted considerable interest, further augmented by the recognition that some benefit also accrues if the remote postconditioning intervention is delayed by 30 minutes after myocardial reperfusion (“delayed remote ischaemic postconditioning”). The biological mechanisms of remote ischaemic postconditioning are unclear, but there appears to be a dependency on several interacting factors, including neuronal and humoral factors as well as transmission of unknown factors via microvesicles (Giricz *et al.*, 2014).

### *Pharmacological postconditioning*

The administration of pharmacological or other biologically active agents during early reperfusion to effect cardioprotection is frequently termed pharmacological postconditioning. For clarity and precision, we believe that the term should be reserved strictly for approaches that recruit or mimic the established pathways associated with ischaemic postconditioning. These approaches would include pharmacological agonists for receptors that are known to participate in ischaemic postconditioning (e.g. adenosine A<sub>2</sub> receptor ligands, or kinin B<sub>2</sub> receptor ligands); or activators of established signal transduction mechanisms participating in ischemic postconditioning (e.g. statins and volatile anaesthetics activating the PI3K/Akt pathway, or NO donors activating the cGMP/PKG pathway). It is usual for administration of such agents to be commenced shortly before reperfusion or immediately at reperfusion onset. A wide variety of agents, unrelated directly to the mechanisms of ischaemic postconditioning, have been reported over many decades as adjuncts to reperfusion. These include calcium channel blockers (Kloner *et al.*, 1991), magnesium salts (Antman, 1995), caspase inhibitors (Mocanu *et al.*, 2000) and adrenoreceptor antagonists (Broadley *et al.*, 2004). Whether or not they are effective at limiting infarct size during reperfusion, such pharmacological treatments should not be described as postconditioning mimetics.

### *Other modified reperfusion approaches*

Several years before the formal description of ischaemic postconditioning it was recognised that modified forms of reperfusion could limit reperfusion injury. Most notable are staged (gradual) reperfusion and acidic reperfusion (see Figure 1). Several

surgical studies in the 1980s showed that gradual, rather than rapid, restoration of coronary blood flow mitigated against the development of reperfusion injuries (arrhythmias and stunning) (Casale *et al.*, 1984; Preuss *et al.*, 1987). This manoeuvre was later shown to limit infarct size (Sato *et al.*, 1997). Similarly, mild acidification of the blood or crystalloid perfusate during early reperfusion showed a similarly protective effect (Inserre *et al.*, 2008). Our understanding of the molecular mechanisms of reperfusion injury has led to speculation that both manoeuvres limit the opening of MPTP during early reperfusion, a mechanism shared in common with the various forms of postconditioning and discussed in more detail below.

## ***Overview of mechanisms of ischaemic postconditioning***

The prevailing conceptual model (see Figure 2) within which the majority of work on ischaemic postconditioning is currently undertaken postulates opening of MPTP during the early minutes of reperfusion as being a critical event leading to cell death. In postconditioned myocardium, a number of complex interlinked signalling pathways are activated by intracellular factors and extracellular autacoids. These signalling pathways ultimately impinge on MPTP, reducing the probability of its opening. This mechanistic framework has been built up through a considerable body of experimental work, including pharmacological and genetic targeting of these pathways, autacoids and components of the MPTP. We will now describe the key evidence supporting the current model, beginning with a discussion of the pivotal role of MPTP.

### ***Mitochondrial permeability transition***

Hunter and Haworth (1979) and Crompton (1987) identified the MPTP as a non-specific channel of defined diameter spanning the mitochondrial inner and outer membranes. More recent work by Halestrap and colleagues made the association between reperfusion and the formation of this pore in an active state. They observed that opening of the MPTP is enhanced by adenine nucleotide depletion, as well as elevated phosphate and oxidative stress, which are biochemical anomalies associated with ischaemia-reperfusion injury (Halestrap *et al.*, 1998). Opening of MPTP permits the passage of molecules up to 1.5 kDa and, with the entry into the mitochondrial matrix of H<sup>+</sup>, results in uncoupling of oxidative phosphorylation, ATP depletion and the onset of cell death by necrosis. Work by Crompton *et al.* (1988) and Griffiths *et al.* (1993; 1995) provided direct evidence of MPTP opening at reperfusion, but not during

ischaemia. Particular features of the intracellular environment in reperfusion appear to contribute to this activation of MPTP. They include oxidising conditions associated with reactive oxygen species (ROS) generation, intracellular  $\text{Ca}^{2+}$  overload and the reversal of intracellular acidosis as a result of  $\text{H}^+$  washout (Buja, 2013). It has been suggested that ischaemic postconditioning and postconditioning mimetic stimuli attenuate opening of the MPTP by reducing intracellular  $\text{Ca}^{2+}$  overload and limiting ROS generation (Leung *et al.*, 2008).

It remains unclear how  $\text{Ca}^{2+}$ , ROS and  $\text{H}^+$  interact with the MPTP, but it has been reported that binding of adenine nucleotide translocase ligands to cyclophilin-D (CYP-D), a subunit of the MPTP, increases sensitivity to  $\text{Ca}^{2+}$ . Mice deficient in CYP-D could not be protected by an ischaemic postconditioning stimulus (Elrod *et al.*, 2010). On the other hand, cyclosporine-A (Cys-A) which inhibits MPTP opening by binding to cyclophilin-D limits infarct size when administered at reperfusion in most animal models tested and in humans (Gedik *et al.*, 2013).

Mitochondrial  $\text{K}_{\text{ATP}}$  ( $\text{MK}_{\text{ATP}}$ ) channels offer another cytoprotective target, through regulating ROS production and  $\text{Ca}^{2+}$  overload. Perfusion with the  $\text{K}_{\text{ATP}}$  channel blocker 5-HD (5-hydroxydecanoate) abolished postconditioning protection in the rat, whereas the  $\text{K}_{\text{ATP}}$  channel opener diazoxide significantly improved cardiac contractile activity (Jin *et al.*, 2012). It is further suggested that intermittent targeting of the  $\text{MK}_{\text{ATP}}$  channel during reperfusion, mimicking postconditioning, affords cardioprotection by ROS compartmentalisation (Penna *et al.*, 2007). Interestingly postconditioning was blocked by administration of an antioxidant during early reperfusion. It is proposed that the *early* generation of ROS may trigger  $\text{MK}_{\text{ATP}}$  channel opening and protein kinase C (PKC) activation which are required for protection, supported by the notion that a channel blocker and PKC inhibitor abrogated protection (Yang *et al.*, 2004).

Subsequent reduction in ROS however may prevent MPTP opening (Clarke *et al.*, 2008).

### *Receptor-mediated mechanisms*

The involvement of a number of extracellular autacoid factors, elaborated or enhanced as a result of ischaemic postconditioning, has been explored extensively. These factors are the subject of a comprehensive discussion elsewhere in this issue (Kleinbongard and Heusch, 2014 this issue) and the interested reader is referred there. In brief outline, the autacoids that have received most attention include adenosine, bradykinin, and opioid peptides. Several studies have demonstrated that ischaemic postconditioning delays the washout of endogenous adenosine and subsequent receptor activation affords protection (Kin *et al.*, 2005). Different receptor subtypes are implicated in different species with A<sub>2A</sub> and A<sub>3</sub> important in rat (Kin *et al.*, 2005), whilst A<sub>2B</sub> signalling is required in the post conditioned rabbit heart (Philipp *et al.*, 2006). Bradykinin B<sub>2</sub> receptors have also been implicated in postconditioning in the rat perfused with the B<sub>2</sub> receptor antagonist HOE140, which abrogated protection (Penna *et al.*, 2007). Interestingly perfusion of bradykinin for 3 min during early reperfusion was unable to afford protection, yet intermittent perfusion in a protocol to match mechanical postconditioning demonstrated comparable infarct limitation. This protocol was unsuccessful when using adenosine. The significance of the protection afforded by bradykinin perfusion in this model remains to be elucidated.

Most recently the opioid receptor has been reported to play a part in postconditioning. The opioid receptor antagonist naloxone abolished the protection afforded by postconditioning alone (Zatta *et al.*, 2008). Similarly to the observations



made with adenosine, postconditioning appears to prevent the washout of pro-enkephalin, suggesting a build-up of endogenous opioid during postconditioning. These observations are supported by recent findings that report that  $\kappa$  opioid receptor activation limits infarct size during early reperfusion, an effect that was blocked by extracellular regulated kinase (ERK)1/2 inhibition (Kim *et al.*, 2011).

### *Protein kinase mechanisms*

The third and most complex element of the postconditioning mechanism is transduction of the extracellular signals described above to the mitochondria, leading to inhibition of MPTP (see above). Signal transduction is via a number of pathways involving protein kinase activation, often sequentially. The discussion below focusses on the major kinases explored to date. While these are grouped discretely for the purposes of this discussion, it needs to be recognised that considerable overlap and cross-talk likely exists between these cascades.

### *RISK pathway (PI3K/Akt and MEK/ERK)*

The RISK pathway, initially described by Yellon's group consists of two related signalling cassettes: PI3K/Akt and MEK/ERK. Both act in a number of biological systems as anti-apoptotic pro-survival signals, classically activated by extracellular ligands including peptide growth factors (Yellon *et al.*, 1999) (see Figure 2). PI3K/Akt and MEK/ERK have been repeatedly demonstrated as major players in mediating the cardioprotective effects of postconditioning in rodent models (Hausenloy, 2009). Tsang *et al.* (2004) reported that Akt was phosphorylated following six 10 sec cycles of reperfusion in the isolated perfused rat heart. Furthermore, endothelial NOS and

p70s6K were also phosphorylated more than in hearts that had undergone a standard reperfusion protocol. These findings were corroborated by observations that the classical PI3K inhibitors wortmannin and LY294002 abolished the protective effect of postconditioning. Subsequently, Yang et al. (2004) reported the importance of MEK/ERK signalling in an isolated rabbit heart model where pharmacological inhibition of MEK/ERK activation abolished the protection. Of note, RISK signalling is implicated in the cardioprotective effect of postconditioning in human atrial muscle *ex vivo* (Sivaraman et al., 2007). Many pharmacological mimetics of postconditioning have been shown to require the participation of either PI3K/Akt or MEK/ERK or both (Hausenloy, 2009).

### *GSK-3 $\beta$*

Inhibition of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) is associated with cell survival and may be considered as a downstream component of RISK signalling. Phosphorylation inhibits GSK-3 $\beta$  activity and thereby inhibits MPTP activity (Juhaszova et al., 2009). However, its relative importance has been disputed in different models. Wagner et al. (2008) reported that both GSK-3 $\beta$  and ERK phosphorylation are significantly increased following postconditioning in rats. These observations were subsequently supported by further biochemical analysis demonstrating increased GSK3 $\beta$  phosphorylation following postconditioning in a rat global ischaemia model. In contrast, GSK-3 $\beta$  double knock-in mice could be protected with a postconditioning stimulus in a global ischaemia model (Nishino et al., 2008). Further evidence is required to ascertain the precise contribution of GSK-3 $\beta$  and how it may contrast in different species.

### *SAFE pathway (JAK/STAT3)*

The survivor activating factor enhancement pathway (SAFE) has been identified as an alternative cytoprotective pathway to RISK that is triggered by tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and JAK/STAT signalling. Lecour's laboratory have reported that pharmacological inhibition of the JAK/STAT pathway reverses the infarct limitation afforded by postconditioning (Lacerda *et al.*, 2009). They also demonstrated that TNF- $\alpha$  signalling through TNFR2 and STAT3 is required to afford protection. The protection afforded was independent of PI3K/Akt and MEK/ERK signaling. TNFR2 antibodies abolished protection afforded by postconditioning whereas TNFR1 knockout mice were still conditioned (Lacerda *et al.*, 2009). Protection observed with TNF- $\alpha$  was not present when the JAK/STAT3 inhibitor AG490 was administered at reperfusion (Lecour *et al.*, 2005). The upstream activators of the SAFE pathway have gathered little attention to date, but it is suggested that autacoids such as those found upstream of the RISK cascades could be involved (Hausenloy *et al.*, 2013). Distal to the SAFE pathway, it is suggested that signalling converges on the mitochondria; however whether the SAFE pathway converges on the same targets as RISK remains to be investigated thoroughly.

#### *cGMP/PKG pathway*

Endogenous NO derived from endothelial nitric oxide synthase (eNOS) is implicated in ischaemic postconditioning in several animal models. Pharmacological inhibition of eNOS activity abolished the protective effects of postconditioning (Tsang *et al.*, 2004). Conversely, many studies have demonstrated the cytoprotective effects of administering an NO donor in the first few minutes of reperfusion although this effect of NO donors is not consistently seen (Bice *et al.*, 2014a). NO activates soluble guanylyl cyclase (sGC) leading to the generation of cGMP and subsequent activation of cGMP-dependent protein kinase (PKG). Several lines of evidence support the

effectiveness of this pathway as a cardioprotective cascade (Krieg *et al.*, 2009; Bice *et al.*, 2014b). In addition, cGMP/PKG signalling through particulate guanylyl cyclase (pGC) targeting via natriuretic peptides has also been demonstrated to afford infarct limitation (Burley *et al.*, 2007). However, at present it is unclear if the PKG pathway is an essential component of ischaemic postconditioning and if it sits alongside the PI3K/Akt pathway or is distal to it (see Figure 2).

### *Anti-apoptotic mechanisms*

The relative contributions that apoptosis and necrosis make in reperfusion injury have long been debated. Specifically the timing of apoptosis during the evolution of myocardial ischaemia/reperfusion injury remains unclear. Sun *et al.* (2009) reported that postconditioning limited myocardial apoptosis in rat neonatal cardiac myocytes. It was reported that TUNEL staining was reduced compared to controls and that ROS generation and intracellular calcium accumulation were reduced. Cytochrome *c* and caspase-3 have also been implicated in postconditioning signalling associated with a reduction in apoptosis. Penna *et al.* (2006) reported that these factors were reduced following postconditioning in an *ex vivo* rat model, whilst increasing the antiapoptotic factor Bcl-2. Inflammatory mediators including cytokines have also been associated with apoptotic regulation. Mechanical postconditioning has been shown to decrease TNF $\alpha$  and limited ROS formation during early reperfusion, resulting in attenuation of apoptosis (Kin *et al.*, 2008). Most recently the apoptosis repressor with caspase recruitment domain has been shown to decrease caspase-3 activity and subsequent apoptosis in chick embryo myocytes following exposure to hydrogen peroxide (Wu *et al.*, 2013).

### ***Clinical studies of ischaemic and pharmacological postconditioning***

From the brief account above it may be inferred that a number of potential approaches exist for the development of postconditioning as a clinical therapeutic intervention. Indeed, as proof of concept, Staat *et al.* (2005) demonstrated that a mechanical postconditioning algorithm could be instituted in patients with AMI with significant reduction in a surrogate marker of infarct size (serum creatine kinase (CK) concentration). Over the last decade further clinical trials of ischaemic postconditioning have been conducted with mixed outcomes. In those studies that measured CK as an endpoint, approximately half of them reported positive outcomes (see Table 1). The remaining trials, all with small cohort sizes, reported neutral or negative endpoints. Most recently a comparatively large trial reported that 4 cycles of 60 second reperfusion and re-occlusion failed to reduce peak CK-MB (Hahn *et al.*, 2013) (see Table 1). A number of potential explanations can be posited for the variability of clinical studies of ischaemic postconditioning. These include variations in postconditioning algorithms. These issues are discussed comprehensively in a recent review (Ferdinandy *et al.*, 2014 in press)

Pharmacological approaches to postconditioning have been assessed in a number of clinical studies. Here we highlight some notable completed studies, related to the mechanisms outlined above.

#### ***Adenosine***

Adenosine was evaluated as an adjunct to clinical reperfusion therapy prior to the formal identification of postconditioning (Mahaffey *et al.*, 1999). A reduction in infarct

size of 33% was demonstrated in patients receiving adenosine prior to thrombolysis and prompted a larger trial in which the primary endpoints were development of congestive heart failure or six month mortality rates (Ross *et al.*, 2005). The results of this 2118 patient trial were disappointing with no significant improvement in primary outcomes. There was however suggestion that in a subset of patients infarct size was reduced in patients who received the highest dose of adenosine. Furthermore, post-hoc analysis suggested that benefit was only observed in patients who received early adenosine treatment (Kloner *et al.*, 2006). Almost half of the patients in the follow up trial underwent angioplasty rather than thrombolysis which also needs to be considered.

#### *cGMP/PKG pathway*

Most recently the results of the NIAMI trial have been published in which nitrite was administered prior to percutaneous coronary intervention (PCI) as a source of exogenous NO (Siddiqi *et al.*, 2014). Extensive experimental studies have demonstrated the protective effects of administering nitrate, nitrite or NO donors prior to reperfusion. Indeed, nitrite has been shown to have vasorelaxant and anti-platelet properties which may be enhanced under ischaemic conditions but these are actions unrelated to a postconditioning effect (Rassaf *et al.*, 2014). *Post hoc* analysis of patients who had been undergoing chronic nitrate therapy were shown to have fewer ST-elevated MI compared to patients who were described as nitrate-naïve (Ambrosio *et al.*, 2010). However, in the NIAMI trial no reduction in infarct size, measured by cardiac magnetic resonance (CMR) imaging was reported in patients receiving sodium nitrite 5 min prior to PCI.

Targeting the cGMP pathway and the  $K_{ATP}$  channel have also been explored in the clinical setting. The large multicentre J-WIND trial treated patients with atrial natriuretic peptide after reperfusion treatment which showed approximately 15% reduction in total CK release. Patients treated with the  $K_{ATP}$  channel opener nicorandil did not show any significant reduction in total CK release (Kitakaze *et al.*, 2007).

#### *MPTP inhibition*

In a small pilot study Cys-A limited infarct size by 20% compared to controls when measured by MRI 5 days after treatment (Piot *et al.*, 2008). Furthermore, no adverse effects of Cys-A were reported. An ongoing multicentre trial (CIRCUS) is further investigating the potential of Cys-A as an adjunct to reperfusion, the primary endpoints being hospitalisation for heart failure and LV remodeling at one year.

#### *Other pharmacological agents*

In addition to exploring pharmacological postconditioning mimetics, other agents that may offer protection in the clinical reperfusion setting have been investigated. Statins, beta blockers, erythropoietin, glucagon-like peptide and glucose-insulin-potassium have all been utilised in clinical trials with varying outcomes. Two small trials in which erythropoietin (EPO) was administered prior to PCI reported conflicting outcomes (Ferrario *et al.*, 2011; Suh *et al.*, 2011). Similar doses were used however a 30 % reduction in CK-MB was reported in one and no improvement was reported in the other. Second and third doses were however administered at 24 and 48 h in the positive outcome trial. The proposed mechanism of action for EPO protection is said to involve inhibition of the myocardial inflammatory response which may have a delayed component explaining the differences in clinical outcomes.

### ***The challenges and opportunities for successful translation***

The picture obtained so far is that myocardial ischaemic postconditioning (e.g. during PCI for AMI) has the potential to limit infarct size but is of variable efficacy. Studies with pharmacological mimetic approaches (e.g. adjuncts to PCI or thrombolysis for AMI) that target the postconditioning signalling pathways described in experimental studies have not been overwhelmingly positive. There are likely to be multiple reasons for these inconsistent findings. They include study design features e.g. (patient inclusion criteria, timing of drug administration); technical limitations to accurate endpoint assessment (e.g. normalised infarct size measurement in humans); attenuation or overwhelming of the postconditioning signalling mechanisms in patients. The latter potentially represents the greatest challenge for successful translation of postconditioning into the therapeutic arena.

### ***The confounding effect of comorbidities***

The majority of experimental studies of ischaemic postconditioning or pharmacological postconditioning mimetics have been performed in healthy, juvenile male animals. These models are devoid of associated risk factors for cardiovascular disease and do not represent the comorbidities often present in the clinical setting. It is now clear that many of the risk factors and comorbid conditions that contribute to or are present in coronary artery disease (senescence, gender-related hormonal background, dyslipidaemia, hypertension, diabetes etc.) modify the signalling pathways underpinning postconditioning (Downey *et al.*, 2009; Przyklenk, 2013; Vander Heide *et al.*, 2013). In experimental models which address these factors, both ischaemic and pharmacological postconditioning effects may be abolished or severely attenuated because of biochemical perturbations brought about by these conditions. The worrying



possibility that the majority of experimental models have not predicted or recapitulated clinical reality might be regarded by some as the killer blow for successful development of clinical postconditioning and may go some way to explaining the massive variability in clinical trials to date. However, we are not so pessimistic. It seems plausible that at least with some of these comorbidities, postconditioning is not abolished absolutely, but rather the threshold for activation of the pathways is raised. For example, in experimental studies where protection by either ischaemic postconditioning or Cys-A was abolished in diabetic hearts, combination of both interventions restored protection suggesting that the diabetic heart could be protected if an increased cardioprotective threshold could be met (Badalzadeh *et al.*, 2012). Moreover, in some cases, treatment or resolution of the comorbidity restores postconditioning's effect. For example, in a rabbit model postconditioning alone could not limit infarct size in high cholesterol fed animals. However, administration of pravastatin was able to afford protection in these resistant animals, an effect that was blocked by eNOS inhibition (Andreadou *et al.*, 2012).

#### *The confounding effect of current drug therapies*

Another intriguing possible explanation for variability in clinical postconditioning studies is that many patients are in fact already in a maximally conditioned state as a result of their existing drug therapy. Bell and Yellon (2014) have recently proposed a "success hypothesis" suggesting that many, perhaps the majority of, patients presenting with acute coronary syndromes (ACS) are already conditioned by the polypharmaceutical regimen of drugs that they are already taking. Statins, ACE inhibitors, beta blockers and opioid analgesics are all commonly prescribed to these patients and indeed have all been shown to be cardioprotective or to have conditioning-like properties in the experimental setting. On the other hand, accounts

of the effect of these drugs to inhibit conditioning mechanisms have been made in some studies. The term “hidden cardiotoxicity” has been proposed which suggests that some of the adjunct therapies used may increase the threshold for cardioprotection (Ferdinandy *et al.*, 2014).

### *Clinical trial design and translating postconditioning*

The disparity between the experimental studies and the clinical trial data so far obtained suggests that translation – both from bench to bedside and *vice versa* – needs to be improved. As identified above, experimental study design needs to be refined for further mechanistic studies to represent better the clinical setting. At the very least experimental models in which comorbidities can be simulated should be used following initial mechanistic studies. It is clear that we need to focus on building on the well-documented signalling cascades and the spatial and temporal modifications to signalling in diseased states.

To date, the majority of clinical trials assessing pharmacological postconditioning mimetics have been unsuccessful or of only modest benefit (see Table 1). But their limited success may be explained in two ways. First, the design of the pre-clinical animal experiments may fail to recapitulate the complexities of the clinical situation and this leads to inappropriate target selection. Second, the design of clinical trials needs to account for the massive heterogeneity of the patient population and the currently limited ability to quantify tissue salvage or measure infarct size standardised to risk zone size accurately and reliably. Unlike laboratory species, the clinical population presenting with AMI is a heterogenous mix of high-risk and low-risk patients, those with large infarcts and those with small infarcts. Unlike the laboratory experiment, the ischaemic risk zone size, the duration of the ischaemic episode and

the speed of successful reperfusion are highly variable. Perhaps most importantly, the high degree of standardisation of infarct size measurement required in the experimental laboratory is effectively unachievable in the clinical setting with presently available methods.

Thus, it seems unlikely that we will achieve a postconditioning intervention that guarantees benefit for all. Much more likely is that an agent which is safe and easy to administer as a single dose – probably a repurposed drug such as Cys-A - could be given to all AMI patients undergoing reperfusion with the expectation that a proportion might benefit. Given the very large number of patients undergoing reperfusion therapy, the global benefit of such an approach could be large.

### ***Author contribution***

Justin Bice drafted the MS and edited the final version.

Gary Baxter drafted the MS and edited the final version.

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**Table 1. Clinical trials utilising mechanical and pharmacological postconditioning in patients presenting with STEMI**

Study/Reference	Treatment protocol	n number Tx/Control	Effect
<b>IPOC</b>			
Laskey 2005	2 cycles of 90 s R/I	10/7	Improved ST-segment resolution, no difference in peak CK.
Staat <i>et al.</i> 2005	4 cycles of 60s R/I	16/14	Improves ST-segment resolution, reduction in 72 h CK.
Ma <i>et al.</i> 2006	3 cycles of 30 s R/I	49/45	Improved WMSI, endothelial function, reduced CK (NS).
Yang <i>et al.</i> 2007	3 cycles of 60 s R/I	23/18	27% reduction in 72 h CK.
Laskey <i>et al.</i> 2008	2 cycles of 90 s R/I	13/11	Improved ST-segment resolution. Improved coronary flow velocity.
Thiabault <i>et al.</i> 2008	4 cycles of 60 s R/I	17/21	40% reduction in CK. 47% reduction in troponin 1.
Zhao <i>et al.</i> 2008	3 cycles of 30 or 60 s R/I	49/26	Reduced apoptosis at 7 days.
Lønborg <i>et al.</i> 2010	4 cycles of 60 s R/I	59/59	19% relative reduction of MI at 3 months. 31% increase in myocardial salvage.
Sörensson <i>et al.</i> 2010	4 cycles of 60 s R/I	38/38	Neutral outcomes.
Xue 2010	4 cycles of 60 s R/I	23/20	Reduction in infarct size (SPECT).
Garcia <i>et al.</i> 2011	4 cycles of 30 s R/I	22/21	Reduction in CK and improved LVEF.
Freixa 2012	4 cycles of 60 s R/I	39/40	No improvement in infarct size (CMR) or LVEF.
Tarantini <i>et al.</i> 2012	4 cycles of 60 s R/I	39/39	No reduction in infarct size at 30 days (CMR).
Thury 2012	4 cycles of 60 s R/I	25/25	Reduction in infarct size (CMR) and CK.
Dwyer 2013	4 cycles of 30 s R/I	51/51	NS reduction in infarct size (CMR).
Hahn <i>et al.</i> 2013	4 cycles of 60 s R/I	350/350	No improvement in ST-segment resolution.
<b>PI3K/Akt</b>			
Nikolaidis <i>et al.</i> 2004	GLP-1 infusion for 72 h post-PCI	10/11	Improved LVEF.
Lønborg <i>et al.</i> 2012	Exenatide 0.12 µg/min 15 min pre-PCI for 6 h	54/51	Reduced infarct size at 90 days (CMR).
<b>Erythropoietin</b>			
Ferrario <i>et al.</i> 2011	33000 IU bolus EPO pre-PCI, then 24, 48 h later	15/15	30% reduction in CK-MB.
Suh <i>et al.</i> 2011	50 IU/kg EPO bolus pre-PCI	29/28	No improvement in CK, CK-MB or CMR at 96 h.
CREATE-ECLA 2005	GIK infusion for 24 h	10091/10110	Neutral outcome.
Kim <i>et al.</i> 2010	80 vs. 10 mg oral atorva-statin pre-PCI	86/85	No difference in CK-MB or troponin.

<b>GPCR</b>			
Ross <i>et al.</i> 2005	70 µg/kg/min adenosine for 3 h within 15 min R	713/703	Reduction in infarct size (SPECT) in sub study (n=243).
Kloner <i>et al.</i> 2006	50 or 70 µg/kg/min adenosine within 15 min R	716/350	Reduction in 1 and 6 month mortality.
<b>cGMP/PKG</b>			
Kitakaze <i>et al.</i> 2007	72 h carperitide infusion post-PCI	277/292	15% reduced total CK. Improved reperfusion.
Siddiqi <i>et al.</i> 2014	70 µmol IV sodium nitrite over 5 min pre-PCI	118/111	No reduction in infarct size (CMR).
<b>Mitochondria</b>			
Kitakaze <i>et al.</i> 2007	Nicorandil IV bolus then 24 h infusion	276/269	No difference in total CK or LVEF.
Piot <i>et al.</i> 2008	Cyclosporine-A 2.5 mg/kg IV 10 min pre-PCI	30/28	Reduction in total CK. 27 patients 20% reduction in MI size (CMR).
Mewton <i>et al.</i> 210	Cyclosporine-A 2.5 mg/kg IV 10 min pre-PCI	28/0	24% reduction in MI size (CMR).

R/I: cycle of reperfusion and ischaemia, CK: creatine kinase, CK-MB: creatine kinase assay, MI: myocardial infarction, LVEF: left ventricular ejection fraction, CMR: cardiac magnetic resonance, MRI: magnetic resonance imaging, WMSI: wall motion score index, SPECT: single-photon emission computer tomography, NS: not significant.

**Table 1.** Clinical trials utilising mechanical and pharmacological postconditioning in patients presenting with STEMI R/I: cycle of reperfusion and ischaemia, CK: creatine kinase, CK-MB: creatine kinase assay, MI: myocardial infarction, LVEF: left ventricular ejection fraction, CMR: cardiac magnetic resonance, MRI: magnetic resonance imaging, WMSI: wall motion score index, SPECT: single-photon emission computer tomography, NS: not significant.

(Nikolaidis *et al.*, 2004; Laskey, 2005; Ross *et al.*, 2005; Staat *et al.*, 2005; The, 2005; Kloner *et al.*, 2006; Ma *et al.*, 2006; Kitakaze *et al.*, 2007; Yang *et al.*, 2007; Laskey *et al.*, 2008; Piot *et al.*, 2008; Thibault *et al.*, 2008; Zhao *et al.*, 2009; Kim *et al.*, 2010; Mewton *et al.*, 2010; Sörensson *et al.*, 2010; Xue *et al.*, 2010; Ferrario *et al.*, 2011; Garcia *et al.*, 2011; Suh *et al.*, 2011; Freixa *et al.*, 2012; Lønborg *et al.*, 2012; Tarantini *et al.*, 2012; Thuny *et al.*, 2012; Dwyer *et al.*, 2013; Hahn *et al.*, 2013; Siddiqi *et al.*, 2014)



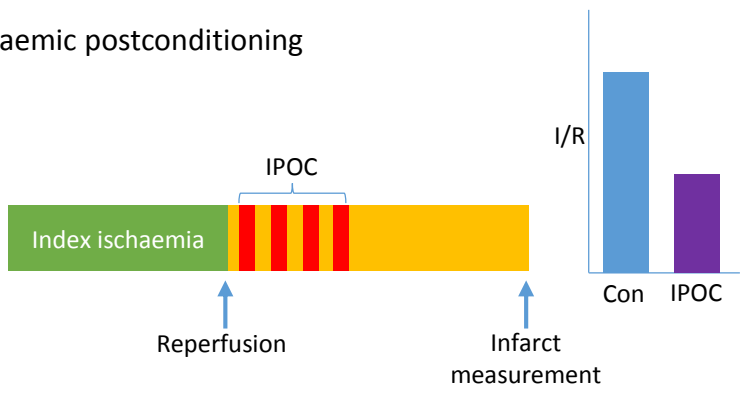
## ***Figures legends***

Figure 1. Schematic representation of myocardial postconditioning protocols and reported infarct limitation afforded by these interventions. Index ischaemia, typically of 30 min duration is followed by intermittent reperfusion-reocclusion of the coronary artery-Ischaemic postconditioning. Similarly when the reocclusion cycles are delayed by as little as 60 s, infarct limitation is no longer afforded. Pharmacological postconditioning typically involves the administration of a postconditioning mimetic during early reperfusion. Remote postconditioning is afforded by occlusion-reperfusion cycles of an artery distal to the myocardium, typically a limb. Modified reperfusion is initiated by gradual reperfusing the occluded area of the myocardium over several seconds. Temporarily reducing the pH during the first minutes of reperfusion can also afford infarct limitation. I/R- infarct to risk zone size, IPOC- ischaemic postconditioning, GrR- gradual reperfusion, AcR- acidified reperfusion, RPOC- remote postconditioning, BNP- brain natriuretic peptide, BAY- cGMP elevating compounds.

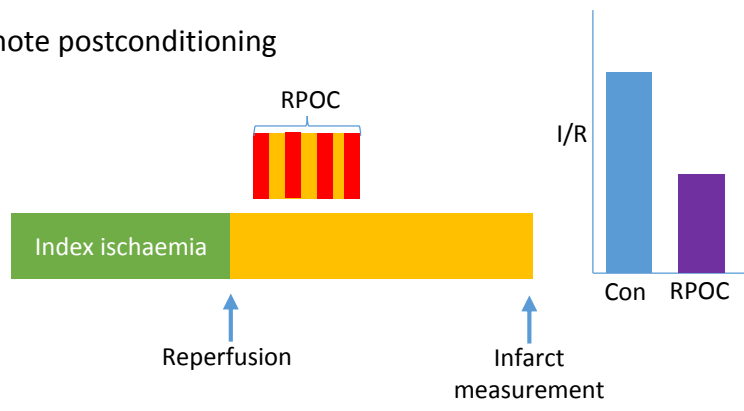
Figure 2. Schematic representation of the identified components of postconditioning signalling in the myocardium. Autacoid factors such as adenosine and opioids along with other extracellular factors initiate cytoprotective signalling through their sarcolemmal receptors. Three distinct signalling pathways have been reported, including RISK, which involves PI3K/Akt and ERK and distal inhibition of GSK-3 $\beta$ . cGMP/PKG signalling through natriuretic peptides and sGC activation is identified as an additional pathway distally targeting mitochondrial potassium channels. SAFE whose major components are TNF $\alpha$  and JAK/STAT has also been demonstrated to play a role in postconditioning. Although described as distinct pathways, their cytoprotective actions are demonstrated to culminate on the mitochondria, specifically inhibition of the MPTP. It remains to be fully investigated as to what extent these pathways interact and colocalize. TNF-R – TNF receptor, RTK – receptor tyrosine kinase, GPCR – G-protein coupled receptor, NPR – natriuretic peptide receptor, JAK – Janus kinase, STAT3 - Signal Transducer and Activator of Transcription, PI3K - Phosphatidylinositol-4,5-bisphosphate 3-kinase, Akt – protein kinase B, ERK – extracellular regulated kinase, eNOS – endothelial nitric oxide synthase, sGC – soluble guanylyl cyclase, cGMP – 3',5'-cyclic guanosine monophosphate, PKG – cGMP dependent protein kinase, SERCA – sarcoplasmic/endoplasmic reticulum calcium ATPase, SR – sarcoplasmic reticulum, MK<sub>ATP</sub> – mitochondrial K<sub>ATP</sub> channel, GSK-3 $\beta$  – glycogen synthase kinase-3 $\beta$ , CYP-D – cyclophilin D, MPTP – mitochondrial permeability transition pore.

**Figure 1**

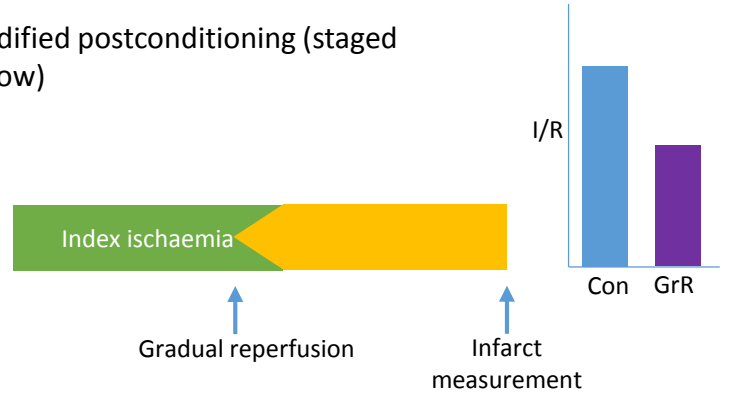
Ischaemic postconditioning



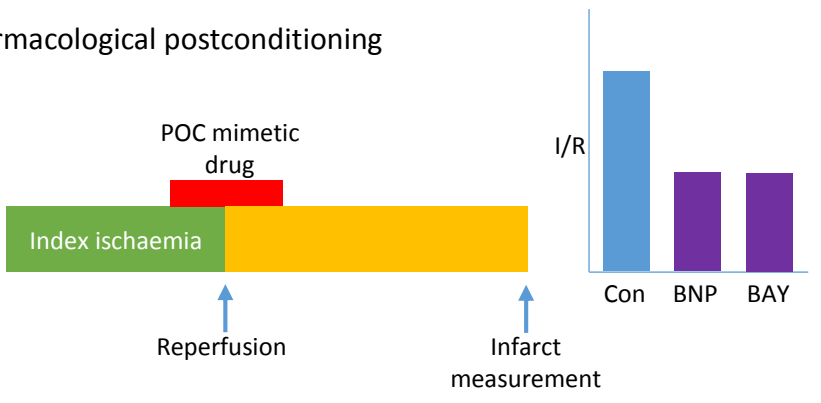
Remote postconditioning



Modified postconditioning (staged reflow)



Pharmacological postconditioning



Modified postconditioning (acidic pH)

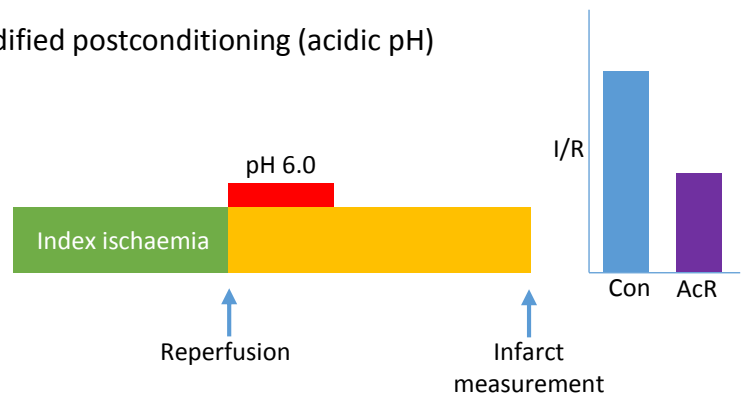


Figure 2

