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REVIEW

Postconditioning and protection from reperfusion injury: where do we stand?

Position Paper from the Working Group of Cellular Biology of the Heart of the European Society of Cardiology

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Ischaemic postconditioning (brief periods of ischaemia alternating with brief periods of reflow applied at the onset of reperfusion following sustained ischaemia) effectively reduces myocardial infarct size in all species tested so far, including humans. Ischaemic postconditioning is a simple and safe manoeuvre, but because reperfusion injury is initiated within minutes of reflow, postconditioning must be applied at the onset of reperfusion. The mechanisms of protection by postconditioning include: formation and release of several autacoids and cytokines; maintained acidosis during early reperfusion; activation of protein kinases; preservation of mitochondrial function, most strikingly the attenuation of opening of the mitochondrial permeability transition pore (MPTP). Exogenous recruitment of some of the identified signalling steps can induce cardioprotection when applied at the time of reperfusion in animal experiments, but more recently cardioprotection was also observed in a proof-of-concept clinical trial. Indeed, studies in patients with an acute myocardial infarction showed a reduction of infarct size and improved left ventricular function when they underwent ischaemic postconditioning or pharmacological inhibition of MPTP opening during interventional reperfusion. Further animal studies and large-scale human studies are needed to determine whether patients with different co-morbidities and co-medications respond equally to protection by postconditioning. Also, our understanding of the underlying mechanisms must be improved to develop new therapeutic strategies to be applied at reperfusion with the ultimate aim of limiting the burden of ischaemic heart disease and potentially providing protection for other organs at risk of reperfusion injury, such as brain and kidney.

Keywords Postconditioning • Ischaemia • Reperfusion

1. Historical development of the concept of infarct size limitation

Since the early 1970s,[1,2] the prognostic impact of the size of an acute myocardial infarction (AMI) has been recognized; infarct size (IS) correlates with arrhythmia severity, the development of heart failure and mortality.[3–5] Hence, the reduction of IS is a worthy goal in the treatment of AMI. Notably, the time-dependent development of infarction and the potential for limitation of experimental IS by various interventions were recognized.

A systematic study by Reimer *et al.*[6] confirmed that necrosis progressed in a 'wavefront' pattern related to the duration of ischaemia, which firmly established the concept that myocardium could be salvaged by initiating reperfusion as early as possible. A microvascular no-reflow phenomenon is associated with, and possibly contributes causally to, the development of AMI and its final extent.[7] Pioneering experimental studies from John Ross's laboratory first demonstrated that early reperfusion could salvage myocardium from infarction and reduce IS.[8,9] This experimental concept of early reperfusion was quickly translated to clinical use, first in Europe[10,11] and then also in

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the USA.[12] Subsequently, early reperfusion therapy was further promoted by the introduction of primary percutaneous coronary intervention (PCI) in myocardial infarction.[13]

Today, reperfusion strategies remain the keystone therapies to reduce IS. Improvements in outcomes have been made through facilitated reperfusion in conjunction with thrombolysis and PCI;[14] no pharmacological agents have been proven to reduce IS clinically. However, they may reduce major adverse coronary events or mortality by other means. Despite better symptom recognition and transport to hospital in order to initiate reperfusion in a timely manner, '. . . it is unlikely that additional substantive improvements in morbidity and mortality can be achieved by reperfusion therapy without . . . new adjunctive therapies'.[15]

1.1 Contribution of reperfusion injury to infarct size

Reperfusion contributes to lethal injury following prolonged periods of ischaemia (*Figure 1A*). The idea of reperfusion injury was first introduced by Jennings *et al.*[16] as significant morphological alterations appearing after the onset of reperfusion, including cardiomyocyte swelling, mitochondrial clarification, amorphous/flocculent densities representing calcium phosphate deposits, hypercontracture, and loss of sarcomere organization. They proposed that reperfusion could hasten the progression of necrosis, but did not suggest that reperfusion caused *de novo* injury. The very existence of lethal reperfusion injury was vigorously debated[17] by those who maintained that lethal injury was expressed or hastened by reperfusion on the one hand,[18] and those who advocated reperfusion as a contributor to *de novo* injury.[19]

The existence of lethal reperfusion injury is strongly supported by evidence of reduced IS achieved by interventions when applied at the onset of reperfusion. Lethal reperfusion injury follows only after severe ischaemic injury and does not occur following fully reversible ischaemic episodes. Reperfusion initiates a cascade of events within the first minutes after restoration of flow, and causes injury in a relatively short time (*Figure 1A* and *B*), leading Piper *et al.*[20] to conclude that 'what happens first must be treated first'. The obvious implication of this concept of reperfusion injury is that more myocardium can be salvaged by adding adjunct reperfusion therapies to early reperfusion.

The mechanisms of reperfusion injury have been under intense investigation for several decades, and have been reviewed elsewhere.[21] Numerous agents and mechanical manoeuvres have demonstrated robust IS reduction when given at reperfusion in experimental studies. However, clinical trials on cardioprotective strategies and drugs were largely negative[22] except for those which will be mentioned below.[15] These negative results may be related, in part, to not applying the intervention at the start of reperfusion, delaying its application beyond the brief window within which salvage is achievable, or ignoring confounders and co-morbidities in animal studies when compared with the clinical reality.

Although the term 'postconditioning' was first introduced by Na *et al.*[23] with respect to prevention of arrhythmias, Zhao *et al.*[24] were the first to report in 2003 the application of postconditioning to limit lethal reperfusion injury in experimental AMI. Postconditioning differed from its preconditioning counterpart in that the mechanical intervention focused exclusively on events occurring during reperfusion (*Figure 2*) and identified the early moments of reperfusion as a key therapeutic window. Later studies by Zhao *et al.* and others[24,25] revealed that postconditioning also reduced cardiomyocyte apoptosis and contracture, coronary endothelial dysfunction, microvascular injury, tissue oedema, and organelle dysfunction. We now know that postconditioning reduces lethal reperfusion injury in multiple species including humans, and also in organs other than the heart, such as brain and kidney.

2. Models and algorithms of ischaemic postconditioning

Two recent analyses have systematically addressed models and algorithms of ischaemic postconditioning.[26,27] For translation to humans, the temporal and spatial evolution of myocardial infarction is of critical importance. Myocardial infarction develops over time, with close dependence on the residual flow through the occluded artery and collateral blood flow. In rodents, infarction develops more quickly than in larger mammals such as dogs and pigs.[28] Primates exhibit a surprising resistance against infarct development.[29,30] In humans, infarct development—assessed by enzyme release—falls in between the time course observed in dogs and pigs.[31] As in humans, preferentially subendocardial infarction is seen in dogs, pigs, and primates, while in mice, infarction occurs preferentially in the outer layers. It is obvious that more reductionist experimental approaches, e.g. isolated, buffer perfused hearts, isolated pieces of tissue, isolated cells, or even isolated mitochondria are even further away from the true translational endpoint, i.e. IS in humans.

2.1 Duration of index ischaemia and protection

The duration of ischaemia—in addition to risk zone size and the extent of residual blood flow—is a critical determinant of IS, and also determines the reduction of IS by postconditioning. In rats after an index ischaemia of 60 min or longer, IS reduction by ischaemic postconditioning was largely diminished. In contrast, with the same postconditioning algorithm applied after an index ischaemia of 30 or 45 min, there was still significant IS reduction.[26] Also, with too brief index ischaemia, postconditioning failed to reduce IS in rats[32] and pigs.[33] In one study in rats with very short duration of index ischaemia, postconditioning even slightly increased IS[34] (*Figure 1B*).

2.2 Postconditioning manoeuvre and protection

The postconditioning algorithm consists of at least three factors: the delay after which the first re-occlusion is established; the duration and number of re-occlusions; and the duration of the interspersed reperfusion. There is consensus that the delay in applying the first re-occlusion can only be short, but the available data are surprisingly sparse. In rats *in vivo*, IS reduction by postconditioning was lost when the first re-occlusion was shifted from 10 to 60 s reperfusion.[35] In rabbits *in vivo*, protection was achieved when the postconditioning manoeuvre was initiated at 30 s reperfusion but was lost at 60 s[36] or 10 min[37] reperfusion. However, in several studies in mice, rabbits, dogs, and also in studies in patients, ischaemic postconditioning still reduced IS when the delay to the first

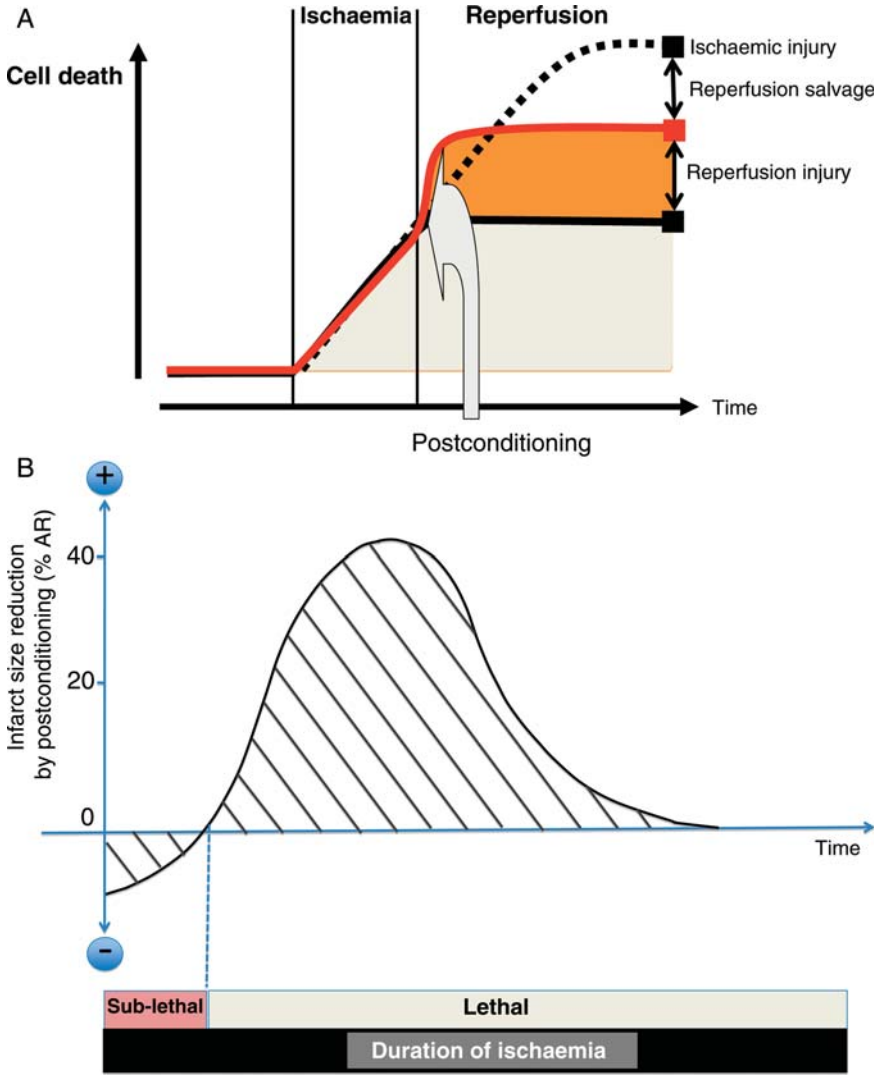

Figure 1 (*A*) The concept of lethal reperfusion injury. During ischaemia, irreversible cell injury leading to cell death occurs within the ischaemic risk zone in a time-dependent manner. In the absence of reperfusion, ischaemic injury would progressively kill more and more cells, eventually accounting for near total cell death (broken line). Reperfusion halts the process of ischaemic cell death but in its early stages imposes injury that results in further cell death, beyond that due to the ischaemic period: this is lethal reperfusion injury. The net result, however, is that the reperfused tissue sustains less cell death than would occur in ischaemic tissue without reperfusion. Hence, targeting cell death due to reperfusion has the potential to maximize cell salvage. Postconditioning applied at the onset of reperfusion limits the extent of reperfusion injury and maximizes reperfusion salvage. Adapted from Garcia-Dorado and Piper.[209] (*B*) Attenuation of lethal reperfusion injury as a function of the duration of the preceding index ischaemia. Infarct size reduction by protective interventions performed after reperfusion (e.g. ischaemic postconditioning) may vary according to the duration of the preceding ischaemia. Experimental studies show that with prolonged periods of index ischaemia, the potential to minimize tissue salvage (infarct size reduction) may be limited probably because the extent of lethal ischaemic injury is so severe. With briefer lethal periods of ischaemia, interventions at reperfusion are able to confer marked reduction in infarct size. When the index ischaemic event is so brief that no infarction occurs (sub-lethal ischaemia), the application of ischaemic postconditioning at the onset of reperfusion may prove injurious and cause a small degree of infarction.

re-occlusion ranged from 60 to 180 s.[26] A delay of the postconditioning manoeuvre until 5 min of reperfusion was probably the major reason for the failure of the first postconditioning experiments in 1992.[38]

The IS reduction by ischaemic postconditioning clearly depends on the 'stimulus' strength. Too few cycles and/or too brief ischaemia/reperfusion within each cycle fail to reduce IS. Increasing the number of postconditioning cycles in rats *in vivo* from three to six[34] and in pigs *in vivo* from four to eight[39] was needed in some studies to establish protection. In rabbits *in vivo*, the prolongation of the ischaemia/reperfusion cycle duration from 10 to 30 s also established protection.[40]

Whether or not postconditioning provides as powerful protection as preconditioning is unclear at present,[26] and efficacy might be dependent on the postconditioning protocol and the duration of the preceding ischaemia. A technical problem which may result from repeated occlusion of the target vessel, specifically in small animal models, is the failure to achieve complete reperfusion, and such failure of reperfusion may confound any potential protection.[41]

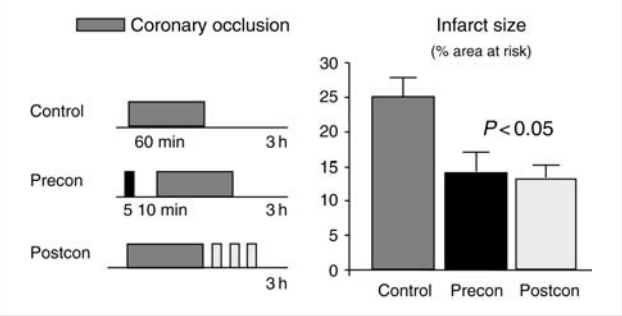

Figure 2 Infarct size reduction by pre- and postconditioning. Coronary occlusion, followed by reperfusion is associated with infarct development in the ischaemic risk zone (Control). Brief periods of ischaemia and reperfusion performed either before index coronary occlusion (Precon: preconditioning) or immediately after index coronary occlusion (Postcon: postconditioning) can significantly reduce infarct size. In animal models such as the dog subjected to coronary artery occlusion and reperfusion, either preconditioning or postconditioning can confer equivalent protection. Data from Zhao *et al.*[24]

2.3 Remote ischaemic postconditioning

The clinical utility of ischaemic postconditioning may be limited by the need to apply an invasive treatment algorithm to the heart itself, and by the requirement to intervene at the onset of myocardial reperfusion. In this regard, remote ischaemic conditioning, in which the heart is protected by applying a conditioning stimulus of one or more cycles of brief ischaemia and reperfusion to a remote organ or tissue, may be preferable.

Kerendi *et al.*[42] first reported that one 5 min cycle of renal artery occlusion/reperfusion applied immediately before myocardial reperfusion could reduce myocardial IS by 50% in an *in vivo* rat model. Myocardial IS limitation, attenuated apoptotic cell death, and less oxidative stress have been observed with remote ischaemic postconditioning using *in vivo* rabbit models in which the protective stimulus has been applied to either the renal artery (three cycles of 30 s reperfusion/occlusions at the end of the index ischaemia),[43] the femoral artery,[44] or the carotid artery (four cycles of 60 s reperfusion/occlusions just prior to myocardial reperfusion).[45] Andreka *et al.*[46] applied the remote ischaemic postconditioning stimulus (four 5 min cycles of hind-limb ischaemia/reperfusion) at the immediate onset of myocardial reperfusion and observed myocardial IS reduction at 72 h using an *in vivo* pig model.

2.4 Postconditioning effects on stunning and reperfusion arrhythmias

Although IS limitation is the primary focus of this review, several studies have examined postconditioning effects on other reperfusion pathologies, namely myocardial stunning and reperfusion arrhythmias. A beneficial effect on stunning is not clearly identified. Different postconditioning protocols failed to improve contractile recovery after brief coronary occlusion not resulting in detectable cell death in dogs.[47] Postconditioning also did not improve contractile recovery in rabbits submitted to more prolonged ischaemic episodes, although it limited IS, suggesting dissociation between the anti-infarct and anti-stunning effects. Even if there is no specific effect of postconditioning on stunning,[48,49] some indirect beneficial effect on ventricular function could be expected from IS limitation. Studies in isolated rat hearts have shown that the benefit of postconditioning on functional recovery was paralleled by its ability to limit necrosis.[50,51] Importantly, the short-term and more long-term beneficial effect of postconditioning protocols which limit IS on left ventricular function has been also observed in humans.[52,53] In an early report, an intermittent form of reperfusion termed 'postconditioning' markedly reduced the incidence of reperfusion-induced ventricular fibrillation (VF) following a 20 min episode of coronary artery occlusion in anaesthetized cats.[23] Several further studies in rats support an anti-arrhythmic action of postconditioning in this species. Galagudza *et al.*[54] reported that in isolated rat hearts displaying persistent VF 15 min after reperfusion, a single 2 min global ischaemic episode resulted in defibrillation. A similar report of the ability of 5 min global ischaemia (instituted 1 min after reperfusion) to terminate reperfusion arrhythmias in the isolated rat heart was reported by Sasaki *et al.*[55] Kloner *et al.*[56] reported that a more classical ischaemic postconditioning protocol (four 20 s episodes) following a 5 min index episode of coronary artery occlusion (insufficient to cause necrosis) in the rat *in vivo* reduced the incidence of ventricular tachycardia during reperfusion. This protective effect of postconditioning was also evident in the senescent (24 months old) rat heart.[57] The mechanism of this anti-arrhythmic effect is unknown: pharmacological inhibition studies suggest that the effect in rat is not mediated by adenosine, PI3-kinase, K_{ATP} channels, or mitochondrial permeability transition pore (MPTP) opening.[58] In pigs subjected to 48 min of coronary occlusion, postconditioning induced a clear reduction in IS, but had no significant effect on reperfusion VF (32 vs. 22%).[33]

2.5 Non-cardiac ischaemic postconditioning

Ischaemic postconditioning has also been shown to confer protection in a variety of non-cardiac tissues and organs including the brain and kidney.

Ischaemic postconditioning was first demonstrated in rat brain by Zhao *et al.*[59] who reported that applying three cycles of 30 s/10 s reperfusion/occlusion to the common carotid artery reduced cerebral IS following an episode of focal cerebral ischaemia. There was attenuated apoptotic cell death and reduced oxidative stress production but the protective effect diminished with increasing ischaemic time. By applying a conventional ischaemic postconditioning protocol of 6 × 10 s reperfusion/occlusion in a rat *in vivo* model of renal ischaemia/reperfusion injury, Liu *et al.*[60] demonstrated reduced renal injury following 45 min ischaemia and 24 h reperfusion and less apoptotic cell death. Renal protection was confirmed in a subsequent study using a more staggered ischaemic postconditioning protocol (comprising 3, 6, and 12 min reperfusion interspersed with 5 min occlusion), findings which were associated with less mitochondrial oxidative stress and improved mitochondrial respiration.[61]

Many of the mechanisms underlying ischaemic postconditioning in brain and kidney are similar to those discussed for the heart below.[62–65]

3. Mechanisms involved in protection by postconditioning

The current mechanistic paradigm for ischaemic postconditioning invokes the activation of signal transduction cascades by autacoid triggers; these accumulate extracellularly in response to the

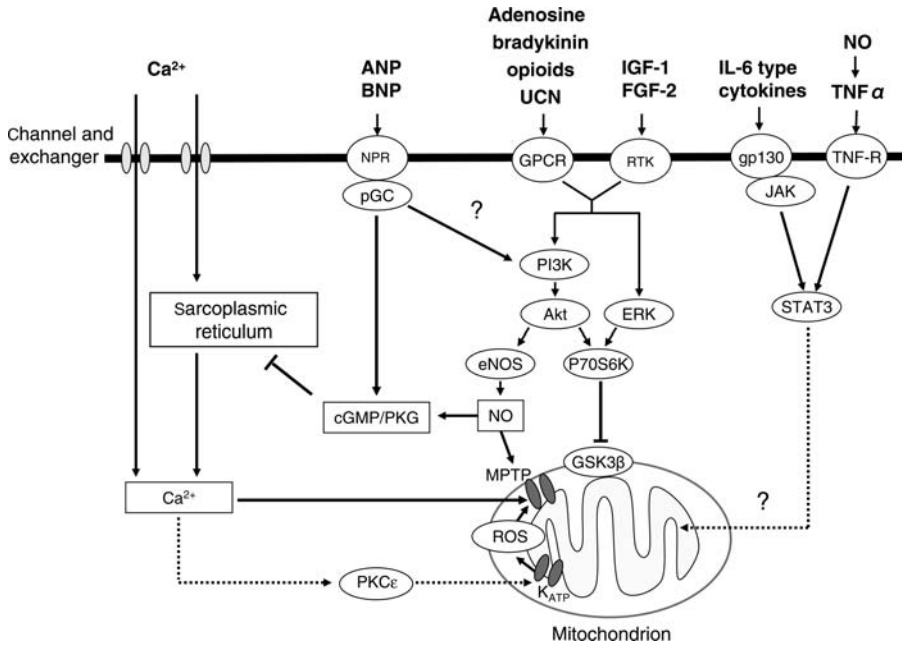

Figure 3 Signalling mechanisms in postconditioning. Several extracellular factors produced endogenously are known to play an essential role in ischaemic postconditioning (adenosine, bradykinin, and opioid peptides). However, other additional autacoids could play a role, since their exogenous administration at reperfusion mimics the effect of ischaemic postconditioning. These include natriuretic peptides (ANP and BNP), peptide growth factors (IGF-1 and FGF-2), and TNF-α. After binding to cell surface receptors, these autacoids promote the activation of kinase signalling pathways. The precise sequence of elements in these pathways and the extent of interaction between different pathways are unclear. However, evidence from some models implicates the activation of PI3K/Akt and p42/p44 ERKs. This pathway, known as the RISK pathway, is proposed to result in inhibition of mPTP opening at reperfusion, via distal components of the cascade which include NO and inhibition of GSK3ß. The extent to which cGMP accumulation and PKG activation contribute to ischaemic postconditioning is not clearly defined at present, but several pieces of evidence support the hypothesis that the activation of cGMP/PKG, either by NO or by other factors such as natriuretic peptides, is protective during reperfusion by attenuating Ca^{2+} cycling which may be a stimulus for mPTP opening. Furthermore, it has been proposed that the activation of an intramitochondrial pool of PKCε might cause opening of the mitochondrial K_{ATP} channel (mitoK_{ATP}), resulting in a slight increase in reactive oxygen species (ROS) formation which eventually causes MPTP inhibition. An alternative pathway, the so-called SAFE pathway, has been proposed to play a role in ischaemic postconditioning. The major components of the SAFE pathway are TNF-α, the kinase JAK which phosphorylates the transcription factor STAT3. It is proposed that after translocation to the nucleus, STAT3 controls the transcription of factors that confer cardioprotection. Also a mitochondrial localization of STAT3 has been suggested; however, both actions of STAT3 need to be finally proven. Key: eNOS, endothelial nitric oxide synthase; GPCR, G-protein coupled receptor; GSK3ß, glycogen synthase kinase-3ß; MPTP, mitochondrial permeability transition pore; ERK, p42/p44 extracellular regulated kinase; NPR, natriuretic peptide receptor; pGC, particulate guanylyl cyclase; PKG, cGMP-dependent protein kinase; RTK, receptor tyrosine kinase; SR, sarcloplasmic reticulum; TNF-R, TNF receptor; ?, unclear at present.

postconditioning stimulus and act on cell surface receptors or other molecular targets (*Figure 3*).

The identification of these mediators owes much to the extensive investigations of the endogenous triggers of ischaemic preconditioning, and it seems possible that, as in ischaemic preconditioning, multiple simultaneous triggers evoke the postconditioning response. The general approaches to investigation have included: assessment of the effectiveness of ischaemic postconditioning under specific pharmacological antagonism or inhibition; modification or loss of postconditioning in receptor- or autacoid-deficient mice; the induction of protection by exogenous autacoids or by selective synthetic agonists when given immediately prior to, or at the onset of, reperfusion.

Definition of the role of an endogenous mediator in ischaemic postconditioning ideally requires satisfaction of all of the following criteria: abolition of the postconditioning effect by specific receptor blockade or by inhibition of the mediator's production; absence of postconditioning in animals, tissues, or cells with genetic disruption of the mediator's production or its receptor(s); induction of a pharmacological postconditioning effect by exogenous administration of the mediator at the time of reperfusion. Increased production or maintenance of extracellular concentrations of the mediator(s) as a direct effect of ischaemic postconditioning might be added to this list although in practice this is the most difficult criterion to satisfy experimentally.

Although isolated cardiomyocytes can be postconditioned, the cellular and/or subcellular origin of most cardioprotective signalling molecules is not firmly known, and it is probably too simplistic to attribute the entire cardioprotective program to cardiomyocytes. In fact, the vascular wall with its endothelium, smooth muscle cells, nerve endings, and (notably in the case of atherosclerosis) inflammatory cells may be a significant source of pertinent signalling molecules: e.g. adenosine,[66,67] bradykinin, and nitric oxide (NO) originate from endothelium and cardiomyocytes, TNF-α originates not only from cardiomyoctes,[68] but also from mast cells and macrophages,[69] and norepinephrine, opioids, and other peptides may originate from perivascular nerves.[70] The formation and release of all these signalling

molecules is probably altered with coronary artery disease and its treatment, including postconditioning. Indeed, postconditioning reduces the activation of the coronary vascular endothelium, the production of pro-inflammatory cytokines, the production of reactive oxygen species (ROS), and adherence of neutrophils to the ischaemic–reperfused coronary artery.[24,25] Preliminary data in abstract form[71] also suggest that postconditioning attenuates the superoxide anion generation by neutrophils in coronary venous blood from the ischaemic–reperfused area at risk. The above discussion supports some role for an anti-neutrophil effect in postconditioning's cardioprotection. The overall picture of reduced neutrophil adherence to the post-ischaemic coronary vascular endothelium, and the reduced accumulation of neutrophils in the infarcted myocardium lend support for an anti-neutrophil effect, although the precise mechanisms are not known at this point. However, postconditioning also reduces IS or cell necrosis in isolated perfused (Langendorff) hearts and in *in vitro* cell culture models, which may argue against a significant anti-neutrophil component in postconditioning's cardioprotection, and raises the question of 'what are mechanisms of injury that are independent of neutrophils that postconditioning addresses in these blood-free models?' In these neutrophil- and red blood cell-free systems, post-ischaemic injury can be attributed, in part, to ROS[72,73] produced by vascular endothelium (NADPH oxidase), cardiomyocytes (xanthine oxidase), and mitochondria (electron transport chain, monoaminooxidases). Since the net effect of oxidants is related to the balance of oxidants and endogenous antioxidants, oxidant-mediated damage may be exacerbated in *in vitro* models by lack of anti-oxidants in plasma (glutathione peroxidase, ascorbate, uric acid, and some amino acids) and red blood cells (glutathione, glutathione peroxidase, catalase, and superoxide dismutase). An IS-sparing effect of postconditioning may be derived from decreased oxidant generation[74–76] and/or increased endogenous antioxidants.[76]

3.1 Autacoid factors and receptor-mediated mechanisms

A number of locally acting mediators of postconditioning, acting in an autocrine–paracrine fashion, have been identified. The best-characterized are classical autacoids that trigger the cardioprotective effects of postconditioning by receptor-mediated mechanisms, namely adenosine, opioids, and bradykinin. There is also less comprehensive evidence in *in vivo* models for the participation of other locally produced endogenous triggers that contribute to protection by postconditioning. These include ROS, NO, reactive nitrogen species,[77] calcitonin gene-related peptide, hydrogen sulfide (H_2S), and epoxyeicosatrienoic acids. Finally, there is a diverse group of naturally occurring mediators that can produce a pharmacological postconditioning effect when given exogenously but with little evidence of participation of the native ligands in ischaemic postconditioning. These include natriuretic peptides, adrenomedullin, erythropoietin, adipocytokines (e.g. apelin, visfatin, and leptin), insulin, peptide growth factors (e.g. transforming growth factor-β, and insulin-like growth factor), and urocortins.

The present discussion will be limited to evidence implicating adenosine, opioids, and bradykinin in the injury-limiting effects of postconditioning.

3.1.1 Adenosine

Interstitial adenosine is increased during ischaemia and is washed out during reperfusion.[78] Multiple adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3) are expressed in the myocardium and the coronary vasculature. For many years, the ability of adenosine and selective receptor agonists to limit lethal reperfusion injury has been controversial.[79,80] Recent research has identified a role of endogenous adenosine in ischaemic postconditioning and has renewed the interest in the potential benefits of adenosine receptor activation at reperfusion.

3.1.1.1 Antagonist and receptor deletion approaches

In mouse hearts, postconditioning delayed the adenosine washout during early reperfusion and enhanced myocardial interstitial accumulation of the nucleoside.[81] In rat hearts, involvement of A_2 and A_3, but not A_1, receptor activation was demonstrated by the use of receptor-selective pharmacological agents.[81] In rabbit hearts, postconditioning was abolished with the A_1 receptor antagonist DPCPX,[82] consistent with a report that postconditioning was abolished in mice with targeted deletion of A_1 receptors.[83] However, other studies in rabbits demonstrated that although non-selective adenosine receptor antagonism abolished protection by postconditioning,[84] selective A_{2B} receptor activation, but not A_1 or A_{2A} activation was involved.[85] In mouse hearts, the selective A_{2A} receptor antagonist ZM-241385 abrogated postconditioning-induced improvement in functional recovery, and postconditioning failed to improve functional recovery in mouse hearts with targeted deletion of A_{2A} receptors.[86] In isolated rat hearts, the protection achieved by the adenosine A_1/A_2 agonist NECA at reperfusion was mediated through both A_{2A} and A_{2B} receptor activation, as evidenced by abrogation of protection with selective A_{2A} and A_{2B} antagonists.[87]

3.1.1.2 Pharmacological postconditioning with adenosine and selective agonists

There is no consistency of an IS-limiting effect in studies using adenosine or A_1 receptor agonists.[79,80] There is, however, a more concordant experimental literature on the ability of A_2 receptor agonists at reperfusion to limit IS among several species. In a recent study in isolated rat hearts, both a selective A_{2A} and a selective A_{2B} agonist reduced IS, and their effect was additive.[87] Some uncertainty is related to the mixed pharmacological profiles of many adenosine receptor agonists. Additionally, several studies have confirmed the protective effect at reperfusion of selective A_3 receptor agonists such as IB-MECA and chloro-IB-MECA in various models.

Thus, a general role of adenosine receptor activation by endogenous and exogenous adenosine has been demonstrated using a combination of pharmacological antagonism and gene deletion, but the relative contribution of different adenosine receptor subtypes remains unresolved and might be species- and model-dependent.[80]

3.1.2 Opioids

Opioid peptides, including met-enkephalins, leu-enkephalins, and dynorphins, and three major opioid receptors (μ-, δ-, and κ- subtypes) are present in myocardium. Ischaemic postconditioning maintained myocardial opioid peptide concentrations during early reperfusion.[88] In rats, the non-selective opioid receptor antagonist naloxone or selective antagonists of δ-, κ-, or μ-opioid receptors abrogated protection by postconditioning.[88,89] Conversely, pharmacological postconditioning was achieved with morphine (non-selective agonist);[88,90,91] selective δ-opioid,[90,92,93] or κ-opioid receptor agonists.[93] Chen *et al.*[91] implicated κ-opioid receptor activation, but not δ-opioid receptor activation, in morphine-induced postconditioning.

Taken together, opioid receptor activation contributes to the effect of ischaemic postconditioning in rodents. Exogenous administration of opioid receptor agonists induces a pharmacological postconditioning effect, independent of the opioid receptor subtypes stimulated.

3.1.3 Bradykinin

Bradykinin is a peptide mediator of the kinin family produced from precursor kininogens by vascular and cardiac endothelium. Two bradykinin receptor subtypes, B_1 and B_2, are recognized of which B_2 is constitutively expressed in cardiovascular tissues, while B_1 is up-regulated under hypoxic and inflammatory conditions.[94] The B_2 receptor antagonists icatibant or WIN64338 abrogated protection by postconditioning,[95] and this finding was confirmed by ablation of B_2 receptors in knockout mice; interestingly, there was partial attenuation in B_1 receptor knockout hearts as well.[83] Conversely, administration of bradykinin at reperfusion limits IS in mouse, rat, and rabbit hearts.[95–98] The mechanisms underlying the protective effect of bradykinin-induced postconditioning are unclear, but participation of both NO and prostaglandin (PGI_2) synthesis has been described in rodent myocardium.[97,98] Thus, endogenous and exogenous bradykinin can mediate protection by postconditioning through the activation of both bradykinin receptors.

Taken together, ischaemic postconditioning enhances the concentration of endogenous autacoids during early reperfusion. The enhanced autacoid concentrations, through activation of their specific receptors, contribute to the reduction in lethal reperfusion injury. Blocking the effect of any of the endogenous autacoids abrogates protection by ischaemic postconditioning while exogenously administering each autacoid is capable of inducing postconditioning-like protection.

3.2 Ionic homeostasis in protection by postconditioning

As is the case with autacoid accumulation, myocardial ischaemia causes accumulation (increased production/reduced washout) of protons and subsequently a progressive fall in intra- and extra-cellular pH, usually within a few minutes. Reperfusion removes extracellular protons and corrects intracellular acidosis, mainly through the activity of the sarcolemmal Na^+/H^+ exchanger (NHE1) and the Na^+-bicarbonate co-transporter. The activity of both transporters contributes to intracellular Na^+ accumulation—and subsequently Ca^{2+} overload—as the increased cytosolic Na^+ concentration stimulates the reverse-mode activity of the sarcolemmal Na^+/Ca^{2+} exchanger (NCE).[99,100] Studies in isolated cardiomyocytes identified the rapid correction of intracellular acidosis as an important determinant of reperfusion injury, which favours Na^+ and Ca^{2+} overload,[101] but also allows the activation of systems that remain otherwise inhibited during low pH (see below).[102] The time course of correction of the intracellular Ca^{2+} homeostasis and tissue pH during reperfusion decides between cell death (recovery of pH occurs first) and survival (recovery of Ca^{2+} occurs first).[100] The rapid normalization of the intracellular Ca^{2+} concentration is critically dependent on the rapid recovery of the activity of the sarcolemmal Na^+/K^+ ATPase upon re-energization.[103]

The protective effect of acidosis during initial reperfusion is not surprising in view of its effects on many molecular mechanisms of reperfusion injury. During the initial minutes of reperfusion, when Ca^{2+} oscillations between the cytosol and the sarcoplasmic reticulum occur, low pH inhibits contractile activity and hypercontracture, and also reduces gap-junction communication and thereby the spreading of cell death.[20] Moreover, low pH during reperfusion prevents opening of the MPTP (see below),[102] calpain activation, and calpain-mediated proteolysis. NHE inhibition at the time of reperfusion does not delay the recovery of intracellular pH *in vivo* because of the compensating activities of other ionic systems (mainly, the Na^+-bicarbonate co-transporter), which might explain the negative results of NHE inhibition given at reperfusion in AMI patients.[104]

3.2.1 Intracellular pH in postconditioning

Heusch[105] was the first to propose that the effect of postconditioning is secondary to the maintenance of acidosis. Cohen *et al.*[106] provided the first experimental evidence for this hypothesis; acidic reperfusion caused equivalent protection as postconditioning in isolated rabbit hearts, and the protection was related to inhibition of MPTP opening.[106,107]

NMR spectroscopy studies in rat hearts showed that postconditioning delays pH recovery for up to 3 min depending on the postconditioning protocol used.[51] In fact, these studies demonstrated that only postconditioning protocols able to induce a significant delay in pH recovery were able to afford cardioprotection. Furthermore, a close correlation between the delay in pH recovery and the magnitude of myocardial salvage was documented.[51] The pH value and duration of infusion that afforded optimal protection was found to be 6.4 and 3 min, respectively, in perfused rat hearts.[108] Interestingly, acidic infusion for less than 2 min was ineffective,[107] and prolongation of infusion beyond 3 min resulted in progressive loss of benefit. The protocol of acidic intracoronary reperfusion in pigs submitted to transient coronary occlusion was as effective as postconditioning in limiting IS, but was accompanied by an increased risk of ventricular arrhythmias.[33]

3.2.2 Calcium homeostasis and calpains in postconditioning

Despite the recognized importance of free ionized Ca^{2+} in lethal reperfusion injury, there is little information on calcium homeostasis in postconditioning. The cytosolic and mitochondrial Ca^{2+} accumulation following hypoxia and re-oxygenation was reduced by hypoxic postconditioning in isolated cardiomyocytes.[74] On the contrary, mitochondria isolated from postconditioned rabbit hearts following ischaemia/reperfusion contained more total (free plus bound) calcium when compared with mitochondria from non-postconditioned hearts.[109] The controversy might be explained in part by ionic[74] vs. total[109] mitochondrial calcium measurements.

Although the issue of calcium handling in postconditioning is unresolved at present, postconditioning might interfere with Ca^{2+}-dependent proteolysis by calpains. Calpains are ubiquitous cytosolic Ca^{2+}-dependent proteases which act on a large number of substrates including structural myofibrillar and sarcolemmal proteins, and calpain activation contributes to sarcolemmal fragility and cell rupture during reperfusion,[110] to detachment of the Na^+ pump,[110] to cleavage of the anti-apoptotic protein Bid, and activation of the pro-apoptotic Bad/Bax pathway.[111] More recently, calpain has been shown to be a target of postconditioning's protection.[51]

Calpain activation depends on the intracellular pH; calpain activity is maximal at pH close to 7.2 and absent at pH of 6.4.[112] Therefore, it can be assumed that pH normalization during reperfusion contributes to calpain-mediated proteolysis. In fact, a close correlation between the myocardial calpain activity during initial reperfusion and IS in

postconditioned rat hearts exists.[51] The observation that transient acidosis at the time of reperfusion effectively reduces calpain-mediated proteolysis and reperfusion injury suggests that calpain is not activated during ischaemia despite the increased cytosolic Ca^{2+} concentration, and that low pH during ischaemia is responsible for this effect. This opens the possibility of IS limitation by administering calpain inhibitors immediately before reperfusion, as recently shown in rats submitted to transient coronary occlusion.[113]

3.3 Signal transduction by protein kinases

Certain protein kinase cascades are implicated in the transduction of signals from the receptor to the potential end-effector; the major kinase pathways identified so far are discussed below.

3.3.1 Reperfusion Injury Salvage Kinase (RISK) pathway

In 2002, Yellon and co-authors[114] introduced the concept of a pro-survival reperfusion signalling pathway. In a study examining the cardioprotective effect of urocortin they demonstrated that the p42/p44 (ERK 1/2) mitogen activated protein kinase (MAPK)-dependent signalling pathway represented an important survival mechanism against reperfusion injury, and suggested that the heart possessed pro-survival 'Reperfusion Injury Salvage Kinase' (RISK) pathways. Yellon and co-author[115] went on to further expand the concept of the RISK pathway, and subsequently they and others demonstrated that the pharmacological activation of pro-survival kinases, such as PI3 kinase-Akt and ERK1/2, at the immediate onset of myocardial reperfusion reduced IS by 40–50%.[115] This involvement of the kinases was demonstrated using a diverse variety of agents, including G-protein coupled receptor agonists and natriuretic peptides, but also pharmacological agents such as statins. Successive studies have also confirmed the role for Akt and ERK1/2 in the setting of postconditioning in both non-diseased animal hearts, as well as in post-infarct remodelling.[37,116,117] Importantly, *ex vivo* studies using human atrial muscle have now confirmed the cardioprotective role of the RISK pathway in the setting of simulated ischaemia/reperfusion injury.[118]

Although the concept of the RISK pathway has gained widespread attention, it is important to appreciate that it is a broad ranging concept which is inclusive of many prosurvival signalling kinase pathways that are upregulated in response to a stress, such as ischaemia/reperfusion, and it is not limited to ERK1/2 or PI3 kinase-Akt. Glycogen synthase kinase-3β (GSK-3β), a protein kinase linked to the regulation of a variety of cellular functions including glycogen metabolism, gene expression, and cellular survival, could either be considered as a specific downstream target of the RISK pathway or indeed as a component of the RISK pathway. Phosphorylation and thereby inhibition of GSK-3β confers cardioprotection through its potential mitochondrial effects which include the inhibition of MPTP opening[119] (see below) and the control of mitochondrial adenine nucleotide transport through the outer mitochondrial membrane.[120] However, there is conflicting information regarding the role of GSK-3β as an important mediator of postconditioning.[121,122] Mice containing a mutant form of GSK-3β, which cannot be phosphorylated and thereby inhibited, were resistant to the myocardial infarct-limiting effects of postconditioning, suggesting that GSK-3β inactivation is required for postconditioning.[121] In contrast, mice with a mutant form of both GSK-3β and GSK-3α, in which the Akt phosphorylation sites were changed rendering them resistant to inactivation, were still amenable to the myocardial infarct-limiting effects of both pre- and postconditioning, suggesting that GSK-3β and GSK-3α inactivation is not necessary for cardioprotection.[122]

Although most interest has focused on the ERK member of the MAPK—this being the first kinase to be associated with the RISK pathway[114]—two other members of the MAPK family[123] have been examined and debated in the context of cardioprotection, namely JNK and p38MAPK.[124–126] With regard to ischaemic postconditioning, however, the only study to investigate the role of these kinases, in which adult rat cardiomyocytes subjected to simulated ischaemia were used, suggests that these MAPKs are inhibited and that their activation at the onset of myocardial reperfusion is detrimental.[127]

From the evidence presented above, it appears that the RISK pathway is fundamental to myocardial protection. However, recent evidence suggests that there are additional and independent pathways which can also be solicited and available to protect the myocardium from ischaemia/reperfusion injury.[128] In pigs, postconditioning activated Akt and ERK 1/2 but did not reduce IS.[129] In mice and rabbits, protection by postconditioning was associated only with ERK but not with Akt activation.[130,131] Recently, Heusch's group[132] demonstrated that in anaesthetized pigs postconditioning reduced IS. However, this protection was not associated with greater phosphorylation of Akt, ERK, p70S kinase, or GSK-3β than reperfusion *per se*, and the protection was not abrogated by combined pharmacological inhibition of the PI3 kinase or ERK1/2 pathways; also protection by gentle reperfusion was not mediated by RISK activation in pigs.[133]

3.3.2 Survivor Activating Factor Enhancement (SAFE) pathway

The activation of the JAK-STAT pathway has been proposed as an alternative cardioprotective pathway, apart from and in addition to the RISK pathway.[134] Lecour and co-authors has termed this alternative pathway 'Survivor Activating Factor Enhancement pathway (SAFE)'.[135,136] The JAK-STAT pathway conveys extracellular stress signals from cytokine receptors on the plasma membrane to the nucleus. Here an assortment of proteins is transcribed relating to a variety of cellular processes, including those involved in cardioprotection. Pharmacological inhibition of the JAK-STAT pathway at the onset of myocardial reperfusion, or its genetic ablation, can abrogate the infarct-limiting effects of postconditioning.[137,138] However, mice with a cardiac-specific STAT3 deletion were still amenable to the infarct-limiting effects of postconditioning, providing a suitable protocol was used. For example, postconditioning using 5 × 5 s cycles of ischaemia/reperfusion reduced myocardial IS but 3 × 10 s cycles did not.[137] In mice with the same cardiac-restricted STAT3 deletion, a postconditioning protocol consisting of 3 × 10 s cycles of ischaemia/reperfusion improved LV function.[138] Lecour and co-authors demonstrated in isolated mouse hearts that the protective effects of TNF-α depended on both STAT3 and specifically the TNF receptor 2, but not on the TNF receptor 1[139] or on the activation of PI3 kinase-Akt or ERK.[135,136,140] Both the RISK and SAFE pathways seem to converge on the mitochondria which may be the target or end effector for the protection.[70]

3.3.3 Sphingosine kinase

Sphingosine kinase (SPhK), which generates sphingosine-1-phosphate (S1P), regulates cell mitosis, apoptosis, cytoskeletal rearrangement, and survival.[141] Hearts isolated from mice lacking SPhK1 sustained larger myocardial infarcts were resistant to protection by postconditioning, and were unable to demonstrate the activation of Akt and ERK 1/2.[142] Potentially, the S1P formed by SPhK in early reperfusion

moves into the extracellular space and activates the S1P-receptor, which then recruits other components of the RISK pathway. On the other hand, sphingosine is a downstream mediator of TNF-α;[143] therefore, the relation of sphingosine kinase to the other cardioprotective kinase pathways is not really clear at present.

3.3.4 Protein kinases C and G

The information on protein kinases C and G in protection by ischaemic postconditioning is limited at present. Penna *et al.*[144] first demonstrated that a non-specific PKC inhibitor abolished the IS-limiting effects of postconditioning in perfused rat hearts, suggesting that postconditioning required the activation of PKC to confer cardioprotection. A subsequent study by Zatta *et al.*[145] found that protection by postconditioning was abolished by pharmacological inhibition of the PKC-ε isoform in early reperfusion. The mechanism through which postconditioning activates PKC is unclear.

Although protein kinase G (PKG) has emerged as a mediator of cardioprotection,[146] its role in IS limiting in ischaemic postconditioning is unclear. Protection by postconditioning has been described to be sensitive to pharmacological inhibition of the NO-sGC-cGMP-PKG pathway,[84,147] although there might be PKG-independent effects of NO including a wide variety of direct actions on mitochondria.

Thus, in summary, ischaemic postconditioning may alter activation of multiple protein kinase and/or phosphatase signalling pathways during reperfusion. The relative importance of each pathway for protection is still controversial and might depend on the species, model, and protocol used.

3.4 Mitochondria in postconditioning

Mitochondria are both a central target of processes triggered by ischaemia, such as elevation in intracellular calcium and ROS, and a pivotal site for determining the preservation or loss of cell viability. Strategies aimed at protecting against ischaemia/reperfusion damage have focused on mitochondria, especially on the MPTP.[148–150]

The MPTP can be described as a voltage- and Ca^{2+}-dependent, high-conductance channel located in the inner mitochondrial membrane, and sensitive to inhibition by cyclosporine A (CsA). Its opening causes a sudden increase in inner mitochondrial membrane permeability to solutes with molecular masses up to 1500 Da.[151] Oddly, the molecular identity of MPTP has not been elucidated yet. Adenine nucleotide translocase and the voltage-dependent anion channel (VDAC) have been proposed to represent MPTP components. However, genetic studies have demonstrated that MPTP opening can still be observed in mitochondria devoid of these proteins that might modulate MPTP function.[152,153] On the other hand, MPTP opening is facilitated by binding of the matrix protein cyclophilin D (Cyp-D) to the inner mitochondrial membrane in a process modulated by both Ca^{2+} and inorganic phosphate.[151] Notably, Cyp-D binding to the inner mitochondrial membrane is prevented by CsA and other molecules interacting with Cyp-D that are usually described as MPTP inhibitors. However, since increasing Ca^{2+} concentration still results in MPTP opening even in the presence of these molecules, MPTP desensitization describes better the effect of this family of compounds. The same applies to mitochondria devoid of Cyp-D.[154]

MPTP opening causes an immediate collapse of the mitochondrial membrane potential ($\Delta\psi_m$) that is followed by ATP depletion. When opening is prolonged, the initial uncoupling-like effect is rapidly followed by respiratory inhibition caused by the loss of pyridine nucleotides and of cytochrome *c*; the latter is potentially dependent on the rupture of the outer mitochondrial membrane caused by MPTP-dependent matrix swelling. The resulting inhibition of electron flow might explain the increased ROS formation induced by MPTP opening. Since the latter event is favoured by ROS,[155,156] a vicious cycle of injury amplification is likely to be established, especially at the onset of reperfusion. MPTP opening is favoured by mitochondrial Ca^{2+} elevation, depolarization, and increases in ROS and inorganic phosphate. These factors are counteracted by physiological MPTP antagonists, such as elevated values of $\Delta\psi_m$ and high concentrations of protons (H^+), magnesium (Mg^{2+}), and adenine nucleotides, especially ADP.[151] During ischaemia, intracellular acidosis, along with high levels of Mg^{2+} and ADP, overrides the MPTP-promoting conditions established by $\Delta\psi_m$ decrease and increases in Ca^{2+} and inorganic phosphate levels. Conversely, upon reperfusion, the recovery of pH together with a burst in ROS formation in the presence of high matrix concentrations of Ca^{2+} and inorganic phosphate creates optimal conditions for MPTP opening despite the antagonizing effect of $\Delta\psi_m$ recovery.

MPTP opening is prevented by endogenous self-defence mechanisms aimed at maintaining cell viability. This is the case with both ischaemic preconditioning and postconditioning.[157–161] This notion is supported by the following major lines of evidence: (i) ischaemic postconditioning reduces MPTP susceptibility in mitochondria isolated during reperfusion[157] and (ii) treatments that antagonize MPTP opening elicit cardioprotective effects similar to those induced by ischaemic postconditioning.[158,162] The inhibition of MPTP opening by CsA was shown to afford significant cardioprotection against ischaemia/reperfusion injury,[153,155,163–165] and this concept has been successfully translated to the clinical setting.[166]

Signalling pathways activated by postconditioning, in particular the RISK pathway, have been assumed to modulate the open probability of MPTP. However, a relevant piece of information that is still missing is how processes occurring in the cytosol modulate MPTP opening in the inner mitochondrial membrane. This might be through (de)phosphorylation of critical proteins in the outer mitochondrial membrane and/or by translocation of cytosolic proteins into mitochondria. For instance, the phosphorylation status of the VDAC appears to affect adenine nucleotide traffic between mitochondria and cytosol and/or the binding of the anti-apoptotic protein Bcl-2.[167]

The relationship between MPTP and oxidative stress might be modulated by additional processes. In this respect, an interesting observation is that postconditioning protection is abolished by treatment with the antioxidant N-acetyl-cysteine during the initial 3 min of reperfusion, but not during the subsequent phase.[144] This early ROS formation would then trigger protective mechanisms that appear to include the activation of mitochondrial K_{ATP} channel (mitoK_{ATP}) and PKC, since their inhibition by 5-hydroxydecanoate and chelerythrine, respectively, abrogates protection. Although the source and the moieties of ROS, and PKC isoform(s) were not elucidated, mitoK_{ATP} activation might underlie postconditioning-induced cardioprotection[37,95,144] by reducing the susceptibility to MPTP opening. In fact, it has been proposed that the activation of an intramitochondrial pool of PKCε might cause opening of the mitoK_{ATP}, resulting in a slight increase in H_2O_2 formation which eventually causes MPTP inhibition.[168]

Although mitochondria are considered the end effectors of protective pathways, it is likely that differences exist between the modalities through which the various conditioning stimuli affect mitochondrial function and structure. So far the only characterized example of

these possible differences is connexin 43. Mitochondrial connexin 43, which is causally involved in ischaemic preconditioning,[169,170] is not a prerequisite for ischaemic postconditioning.[171]

4. Co-morbidities and co-treatments and postconditioning

Many of the above signalling elements might be affected by confounders, co-morbidities, and co-treatments. For example, the bradykinin concentration is increased by ACE inhibition, the NO/ROS balance is affected by atherosclerosis, and age reduces the expression of protein kinases and STAT3. Therefore, it is of utmost importance to assess the effects of these entities on protection by postconditioning.

4.1 Gender and age as confounders of protection by postconditioning

Whether cardioprotection by postconditioning is gender-dependent is not really clear. After 30 min ischaemia, IS was smaller in female than male hearts. However, postconditioning reduced IS in both genders, though the effect was smaller in females[32] and dependent on the duration of the sustained ischaemia.[172] In a single study in female rats,[173] IS *per se* was smaller than expected from male rats, but ischaemic postconditioning failed to protect and even increased IS.

Age has a major impact on IS reduction by ischaemic postconditioning in mice, but not in rats.[174,175] In isolated hearts from mice older than 80 weeks, postconditioning failed to reduce IS.[131] Postconditioning by three cycles of 10 s/10 s ischaemia/reperfusion reduced IS in young (<12 weeks) mice *in vivo*, but failed to do so in mice older than 52 weeks. However, changing the postconditioning algorithm to five cycles of 5 s/5 s ischaemia/reperfusion in these mice fully re-established the protection.[137] Interestingly, the biological age of these mice was similar to the biological age of patients in which postconditioning still reduced IS considerably. The induction of postconditioning protection may be a question of optimization of the protocol.

4.2 Underlying conditions and protection by postconditioning

Most experimental studies on cardioprotection have been undertaken in young and healthy animals. However, ischaemic heart disease in humans is a complex disorder caused by or associated with known cardiovascular risk factors including smoking, obesity, hyperlipidaemia, diabetes and hypertension, or pre-existing diseases (e.g. heart failure). In addition, patients with CAD vulnerable to myocardial infarction may be on various pharmacological treatments. These conditions are associated with fundamental molecular alterations that can potentially affect the development of ischaemia/reperfusion injury *per se* and responses to cardioprotective interventions such as postconditioning.

Hyperlipidaemia, especially hypercholesterolaemia, is regarded as an independent risk factor for the development of CAD, increases the severity of myocardial ischaemia/reperfusion injury, and interferes with cellular mechanisms of cardioprotection.[176,177] However, little is known about the effect of postconditioning in hyperlipidaemia. Iliodromitis *et al.*[178] showed the loss of the IS limiting effect of postconditioning in rabbits fed a cholesterol-enriched diet for 6 weeks. The loss of the IS limiting effect of postconditioning was confirmed in hearts isolated from chronically cholesterol-fed rats.[77] However, Donato *et al.*[82] found no alteration in the IS limiting effect of postconditioning in rabbits fed 1% cholesterol for 4 weeks. The reasons for the conflicting results are unknown, but might be related to the severity and duration of the disease state and extracardiac pathologies in the different experimental hyperlipidaemia models. Apart from the accumulation of tissue/membrane cholesterol and alterations in NO-cGMP and peroxynitrite signalling,[176,177,179] the exact cellular mechanism by which hyperlipidaemia may influence postconditioning is not known.

Very little is known on the interaction of diabetes with postconditioning. Both diabetic as well as obese mice have been reported to be resistant to protection by postconditioning, a finding that has been associated with insufficient activation of the RISK pathway.[180,181] Similarly, in pre-diabetic rats with metabolic syndrome, sevoflurane-induced postconditioning no longer reduced IS and even CsA given at reperfusion failed to protect the pre-diabetic heart.[182]

In spontaneously hypertensive rats, postconditioning failed to reduce IS.[183] In rats with pressure overload-induced LV hypertrophy, postconditioning was still capable of reducing IS. Also, ischaemic postconditioning, as well as pharmacological postconditioning with isoflurane, was effective in reducing IS and activating the RISK pathway in rat hearts 6 weeks after MI.[117,184]

Although protection by postconditioning was reduced in animals with a single risk factor, whether or not and how much any of these risk factors blunts IS reduction by postconditioning in CAD patients needs to be determined (see below). Acute MI patients treated by postconditioning will have received several pharmacological treatments because of (i) pre-existing risk factors and/or (ii) treatment of AMI; e.g. aspirin, heparin, clopidogrel, β-blockers, morphine, nitrates, etc. Whether these treatments affect IS reduction by postconditioning in patients is currently unknown.

Some pharmacological treatments have been shown to impact on postconditioning's IS reduction in animal models. For example, β-adrenergic signalling is involved in protection by desflurane-postconditioning,[185] and accordingly β-blockade can abolish such protection.[185] Whether or not this holds true for ischaemic postconditioning is currently unknown. On the other hand, ischaemic postconditioning is lost in rat hearts with chronic coronary stenoses, and protection is restored by carvedilol.[186] The protection by the selective bradycardic agent ivabradine is still achieved when given in a postconditioning modality just before reperfusion, and is not dependent on heart rate reduction.[187] Statins may also interfere with IS-limiting effect of postconditioning in rats.[188] Ischaemic (see above) and sevoflurane-induced postconditioning has been shown to be blocked by anti-diabetic K_{ATP}-channel blockers in rats.[189]

5. Clinical application of ischaemic or pharmacological postconditioning

In 2005, the experimentally proven concept of ischaemic postconditioning was translated into the clinical setting (*Figure 4* and *Table 1*). In a proof-of-concept trial, patients with a first-time ST elevation myocardial infarction (STEMI) having chest pain for less than 6 h and a need for angioplasty revascularization underwent direct stenting. Control patients underwent no additional intervention after reperfusion, while postconditioned patients underwent four cycles of 1 min inflation and 1 min deflation of the angioplasty balloon at low pressure within the first minute after stent implantation, with placement of the

balloon just upstream of the stent. Area under the curve of creatine kinase release over the first 3 days of reperfusion (as a surrogate for IS) was significantly reduced by 36% in the postconditioned vs. control group.[190] This study confirmed that lethal reperfusion injury represents a significant amount of the overall myocardial damage in AMI patients, and that this tissue destruction can be prevented by a timely intervention. IS reduction by PCI postconditioning persisted 6 months after AMI (IS assessment by ^{101}Thallium SPECT imaging), and resulted in a significant improvement of contractile function at 1 year after infarction.[191] These findings are in agreement with a retrospective study by Darling *et al.*[192] who reported that AMI patients who underwent four or more balloon inflations–deflations developed smaller infarcts than patients who received less than four of these brief cycles of ischaemia/reperfusion. Similarly, Wang *et al.*[52] reported in a retrospective analysis of a cohort of 433 STEMI patients that those with more than three balloon inflations at the time of reperfusion displayed a lower peak of creatine kinase release, a lower LV end-systolic volume, and a higher ejection fraction, than those with one or two inflations.

Previous experimental studies have indeed clearly demonstrated that staged reperfusion provides some protection,[193,194] although different from that observed with postconditioning (i.e. staged reperfusion protects from stunning, whereas postconditioning does not). To our knowledge, there has been no report directly comparing staged reperfusion vs. ischaemic postconditioning. This issue is of importance in clinical practice, mainly because reperfusion may be obtained using two different techniques, i.e. angioplasty and fibrinolysis. Whether ischaemic postconditioning is efficient when reperfusion is obtained by fibrinolysis is still unknown. This latter mode of restoration of coronary flow may possibly modify the efficiency of postconditioning. Fibrinolysis may cause a more gentle reflow than angioplasty due to both a slower disaggregation of the thrombus, and mainly, the persistence of a coronary artery stenosis which attenuates hyperaemia.[41] Also, while the timing of reflow by angioplasty is sharp, the exact time of reopening of the coronary artery by thrombolysis may be difficult to determine accurately, and it may only be suggested by the reduction of chest pain and some signs on the ECG such as ST-segment shift reduction or the occurrence of some types of arrhythmias. Timing, however, is of major importance since experimental evidence indicates that any protective intervention can be effective only if performed within the first minutes of reflow.

5.1 Remote ischaemic conditioning

Loukogeorgakis *et al.*[195] demonstrated that remote ischaemic postconditioning could be produced in both human volunteers and patients with CAD in a manner which was sensitive to inhibition by glibenclamide (a K_{ATP} channel blocker) using endothelial function, but not IS, to assess protection. In a recent proof-of-concept clinical study, Botker *et al.*[196] have demonstrated that four 5 min cycles of cuff inflation/deflation to the arm during the ongoing ischaemia administered in the ambulance, improved the myocardial salvage index in STEMI patients receiving primary PCI.

5.2 Potential for pharmacological postconditioning

Although coronary angioplasty is largely used for the treatment of AMI, a large number of patients are reperfused by thrombolysis or

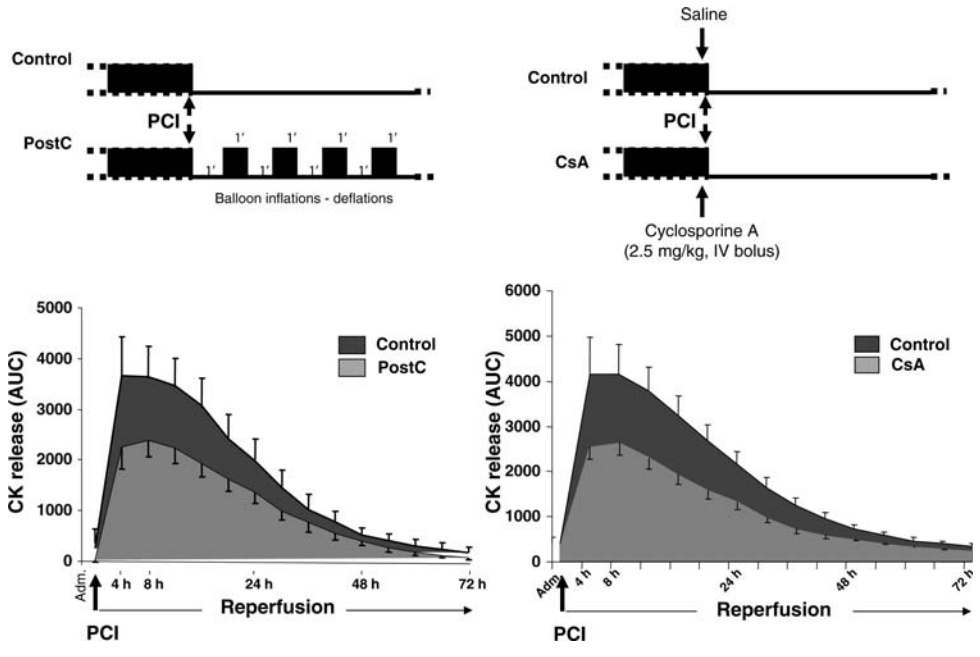

Figure 4 Ischaemic or pharmacological (cyclosporine A) postconditioning in STEMI patients. Left panel (top): STEMI patients treated by PCI (primary coronary intervention) were randomly assigned to receive either no further intervention (control) or four cycles of 1 min angioplasty balloon inflation followed by 1 min balloon deflation, starting less than 1 min after direct stenting. Right panel (top): STEMI patients treated by PCI (primary coronary intervention) were randomly assigned to receive either an IV injection of saline (control) or an IV bolus administration of 2.5 mg/kg of cyclosporine A (CsA). Left and right panels (bottom): area under the curve of creatine kinase (CK) release during the first 3 days of reperfusion in control vs. postconditioned (PostC: left) or cyclosporine A (CsA: right) treated patients.

Table 1 Clinical studies of ischaemic and pharmacological postconditioning in ST-elevation MI patients

Reference	Patient selection	Time (min)	*n*	Protocol	Effect
210	<12 h LAD, RCA, LCx	341	17	2 cycles of 90 s R/I	Improved ST-segment resolution. Improved coronary flow velocity.No difference in peak CK
190	<6 h LAD or RCA	318	30	4 cycles of 60 s R/I	Improved ST-segment resolution. Improved MBG, 36% reduction in 72 h CK
211	<12 h LAD, RCA, LCx	396	94	3 cycles of 30 s R/I	Improved WMSI, endothelial function. Less CK ($P = NS$)
212	<12 h LAD, RCA, LCx	312	41	3 cycles of 30 s R/I	27% reduction in 72 h CK. 27% reduction in MI SPECT 1 week. LVEF from 44 to 54% at 1 week ($P = NS$)
191	<6 h LAD or RCA	283	38	4 cycles of 60 s R/I	40% reduction in CK. 47% reduction in troponin I. 39% reduction in MI SPECT 6 months. 7% increase in LVEF at 12 months
213	<6 h LAD	228	24	2 cycles of 90 s R/I	Improved ST-segment resolution. Improved coronary flow velocity
214	<12 h LAD, RCA, LCx	264	75	3 cycles of 30 or 60 s R/I	Reduced apoptosis at 7 days (serum fas ligand)
215	<12 h LAD, RCA, LCx	255	118	4 cycles of 30 s R/I	19% relative reduction of MI size 3 at months. 31% increase in myocardial salvage ratio. 41% reduction in patients developing heart failure
216	<12 h LAD, RCA, LCx	128	21	GLP-1 infusion for 72 h post-PCI	Improved LVEF
217	<12 h LAD, RCA, LCx	240	20 201	Glucose-insulin-potassium pre-PCI	No beneficial effect
200	<3.17 h LAD	128	1066	50 or 70 mg/kg/min adenosine IV <15 min of PCI	Reduction in 1-month mortality (9.4 vs. 4.2%). Reduction in 6-month mortality (11.2 vs. 7.3%). Reduction in 6-month composite clinical endpoint of death, in-hospital CHF, or rehospitalization for CHF (17.2 vs. 12.0%)
202	<12 h LAD, RCA, LCx	240	569	72 h carperitide (ANP) IV infusion post-PCI	15% reduced total CK. No difference in troponin T. Improved reperfusion
202	<12 h LAD, RCA, LCx	210	545	Nicorandil IV bolus, then 24 h infusion	No difference in total CK or LVEF
218	<6 h LAD	127	94	0.05–5.0 mg IV KAI-9803 pre-PCI	Trend for reduced CK, troponin I, SPECT MI
166	<12 h LAD, RCA	292	58	2.5 mg/kg CsA IV bolus pre-PCI	40% reduction in CK. 13% reduction in troponin I ($P = NS$). 20% reduction in MI by CMR ($n = 27$)
53	<12 h LAD, RCA	292	28	2.5 mg/kg CsA IV bolus pre-PCI	24% reduction in MI size on CMR at 6 months. Smaller increase in LV end-systolic volume
219	<12 h LAD, RCA, LCx	341	30	33 000 IU bolus prior to PCI then 24, 48 h later	30% reduction in CK-MB
220	<12 h LAD	341	57	50 IU/kg EPO bolus prior to PCI	No difference in infarct size by CK, CK-MB, or CMR at 4 days
221	<12 h LAD	241	171	80 vs. 10 mg oral atorva-statin prior to PCI	No difference in infarct size by CK-MB or troponin

Patient selection-time from onset of chest pain. ST-elevation myocardial infarction in LAD (left anterior descending) artery, RCA (right coronary artery), and Cx (circumflex) artery. Time denotes the average time from onset of chest pain to intervention. R/I, cycle of reperfusion/ischaemia; CK, creatine kinase; MBG, myocardial blush grade; WMSI, wall motion score index; MI, myocardial infarction; SPECT, single-photon emission computed tomography; LVEF, left ventricular ejection fraction; PCI, percutaneous coronary intervention; CMR, cardiovascular magnetic resonance; CHF, chronic heart failure; ANP, atrial natriuretic peptide; CsA, Cyclosporine-A; EPO, erythropoietin.

cannot benefit from PCI postconditioning for technical reasons. There is, therefore, an obvious need for a pharmacological mimetic to ischaemic postconditioning (*Table 1*). According to the narrow time window as defined in experimental studies, one has to consider that such a postconditioning drug should be administered before reperfusion, in order to be circulating at the time of onset of coronary reflow. In theory, it could be administered to all patients with ongoing AMI, either as an adjunct to thrombolysis or even just before coronary angioplasty (as an ischaemic postconditioning mimetic).

Clinical trial data from the AMISTAD-I, AMISTAD-II, and ADMIRE trials provide an uncertain picture with regard to the clinical use of adenosine and synthetic adenosine agonist AMP579.[197–199] The largest of these trials, AMISTAD-II, has been comprehensively re-analysed.[200] The administration of adenosine as an adjunct to reperfusion was of greatest benefit in patients receiving early reperfusion [<3.17 h (median) from onset of chest pain]. Benefit was seen in terms of greater early and late survival, and a reduction in the composite endpoint of death or CHF at 6 months. However, it is not clear that this benefit of adenosine treatment was entirely attributable to IS limitation.

A number of other naturally occurring mediators display postconditioning properties when administered exogenously in experimental AMI, although their roles in ischaemic postconditioning are not established. One example which has been translated to clinical trial is the use of natriuretic peptides. Prevention of reperfusion injury by stimulation of the cGMP synthesis was proposed by García-Dorado.[201] In the J-WIND trial, recombinant ANP administration as adjunct to

reperfusion therapy was shown to produce a small but significant reduction in IS, and improved 6-month ejection fraction in a non-selected population of patients with AMI.[202] Although aspects of the trial design were unsatisfactory, the result nevertheless is in general agreement with experimental studies showing the postconditioning potential of natriuretic peptides.[203] Of interest, nicorandil, which activates K_{ATP} channels and releases NO, was without any beneficial effect in the second arm of the J-WIND trial.

Pharmacological inhibitors of MPTP (including CsA) reduce IS in experimental *in vivo* preparations to a similar extent as ischaemic postconditioning,[162,204] making MPTP inhibition an interesting therapeutic target. Recently, Piot *et al.*[166] addressed whether inhibition of MPTP may attenuate IS in AMI patients. They reported that CsA, administered as an intravenous bolus immediately before coronary artery reperfusion by PCI, could significantly reduce IS measured by cardiac enzyme release during the first 3 days of reperfusion and by MRI at day 5 after reflow. This suggests that pharmacological postconditioning can be as efficient as PCI postconditioning, and opens a new therapeutic area for all AMI patients.

The equivalence between ischaemic and pharmacological postconditioning is based on: (i) comparable experimental protocols with the protective intervention being made either immediately before or within the first minute after reperfusion, and (ii) a comparable benefit in terms of IS reduction. In clinical practice, the only available data in the postconditioning era come from the cyclosporine study by Piot *et al.*,[166] which can be compared with angioplasty postconditioning data. The IS reduction was quite similar in these trials. Whether a postconditioning drug may be more efficient than angioplasty postconditioning could be worth looking at in future trials; angioplasty postconditioning could then be considered a reference protective intervention for the potential of IS reduction in AMI patients.

6. Conclusions/positions

(1) There is accumulating evidence that lethal reperfusion injury is reduced by ischaemic or pharmacological postconditioning in animals and in small-size clinical trials.
(2) The evidence remains, however, insufficient to recommend a widespread clinical use of ischaemic or pharmacological postconditioning to reduce IS in clinical practice, and the enthusiasm arising from these encouraging proof-of-concept studies must be tempered by the need to demonstrate an actual clinical benefit in larger scale clinical studies.
(3) The extent of protection by ischaemic postconditioning is dependent on the duration of index ischaemia and the postconditioning protocol used. Species- and inter-individual variations are obvious. Whether the same is true for pharmacological postconditioning is unknown.
(4) The signalling pathway(s) involve triggers, protein kinases, and end-effector(s): the contribution of each of these players may vary depending on the experimental model (cell type, *in-vitro*, *in-vivo*) and, again, the postconditioning stimulus used. More importantly, there appear to be redundant pathways which are activated and interact.
(5) At present, there is insufficient information to judge how and to what extent confounding factors, including the classical CAD risk factors, drug treatments, gender, and age influence postconditioning efficacy.
(6) There are various approaches to conditioning the heart and other organs against reperfusion injury.

7. Perspectives

(1) There is a need to define the optimal protective protocol for a given duration of the index ischaemia.
(2) There is a need to define the interdependence of the signalling pathways (all-or-nothing phenomenon) and the optimal molecular signalling target for protection.
(3) There is a critical need for pre-clinical and clinical studies that examine cardioprotection specifically in relation to complicating disease states and their drug treatments.
(4) There is a critical need for clinical research, including large-multicentre studies, to determine the impact of postconditioning on patient outcomes.

8. Designing future infarct size reduction trials

Before designing clinical studies, it is necessary to perform a thorough analysis of the conditions of success of these positive small-size trials, and put these into perspective with the numerous negative IS reduction studies performed during the past two decades (for review see[22,205]). It would be insufficient to simply transfer a postconditioning regimen to the AMI clinical setting without taking into consideration the major determinants of IS, namely: the timing of application of the protective intervention with respect to the onset of reflow; the duration of ischaemia; the size of the area at risk; and the extent of the collateral circulation.[6]

As mentioned earlier, the time window for ischaemic postconditioning is a critical factor.[35] During PCI postconditioning, the first angioplasty balloon re-inflation was always performed within the first minute after direct stenting of the culprit coronary artery; in the case of CsA-induced protection, the drug was administered before re-opening of the coronary artery. Also, only patients with a fully occluded culprit coronary artery (TIMI flow grade at admission coronary angiography of 0 or 1) were included, thereby eliminating all patients who had undergone spontaneous reperfusion before PCI, i.e. those who had already been exposed to reperfusion injury before the protective intervention.

Taking into account the size of the area at risk is a key issue for all IS reduction studies. The studies by Staat *et al.*,[190] Thibault *et al.*,[191] and Piot *et al.*,[166] who used area at risk as a covariate for analysing IS reduction, demonstrated that the larger the area at risk, the greater the myocardial salvage in patients with AMI. One may even question whether IS reduction by postconditioning is a real issue in patients with a small area at risk, who will anyway develop small infarcts and have a very good cardiovascular prognosis. In contrast, when the area at risk is larger, any additional myocardial salvage becomes clinically relevant. One can expect that postconditioned patients will less often develop adverse LV remodelling and possibly have a lower risk to develop heart failure; this latter issue needs to be addressed in future large-scale trials.[53] Obviously, the technique used to measure area at risk in patients with AMI is critical and remains an unresolved issue in the clinical settings of STEMI.

Although SPECT imaging with ^{99}Tc-sestamibi may be of interest here, its application remains difficult on a daily basis for the care of

AMI patients.[196] Recent pre-clinical reports suggest that T2-weighted MRI is able to measure area at risk, based on the detection of oedema in the early hours after reperfusion.[206] Although very encouraging, this approach might not be appropriate for IS reduction studies since therapeutic interventions that reduce IS, including ischaemic postconditioning, also reduce reperfusion oedema.[207,208] Any strategy based on the assessment of oedema may then underestimate the size of area at risk in the treated, but not in the control group, and thus bias the evaluation of myocardial salvage. Clearly, further work is needed to find the appropriate technique to assess this major determinant of IS in AMI patients.

Collateral flow is the third major determinant of IS. Patients with visible collaterals at admission coronary angiography are protected and develop smaller infarcts even in the absence of any protective interventions. Since it remains almost impossible to assess myocardial blood flow in acute clinical situations, these patients should be excluded from trials exploring the efficiency of IS-reduction interventions. Interestingly, when considering these confounding factors, the patients who benefitted most from the therapeutic intervention (postconditioning or CsA) were those who displayed larger areas at risk, lower collateral flow, and a fully occluded coronary artery at admission.[166,196] Taking into account these major determinants of IS into the design of future trials will help us improve their accuracy and power to explore future treatments aimed at preventing lethal reperfusion injury.

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