Orphan targets for reperfusion injury

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Cardiomyocyte death secondary to transient ischaemia occurs mainly during the first minutes of reperfusion in the form of contraction band necrosis. Research on the mechanisms leading to sarcolemmal rupture and necrosis during initial reperfusion identified several promising pharmacological targets directed either to correct the alterations in Ca\(^{2+}\) handling occurring during this period (\(\text{Na}^+\)/\(\text{H}^+\)-exchanger, reverse mode of \(\text{Na}^+\)/\(\text{Ca}^{2+}\)-exchanger, sarcoplasmic reticulum) or to interfere with its consequences [hypercontracture, calpain activation, and mitochondrial permeability transition pore (mPTP) opening]. However, despite the fact that pharmacological tools against some of these targets have consistently demonstrated that it is possible to reduce infarct size in experimental studies by interventions applied at the time of reperfusion, the translation of these approaches to clinical practice has failed due in part to the lack of drugs able to be tested in humans. Recently, the benefits of both post-conditioning and inhibition of mPTP have been supported by proof-of-concept trials demonstrating the clinical applicability of strategies aimed at preventing lethal reperfusion injury. These promising results should stimulate efforts to develop drugs testable in humans against known, unexploited targets involved in reperfusion injury and to identify and validate additional ones.

1. Introduction

In patients with ST-segment elevation acute myocardial infarction (AMI), the main therapeutic goal is to restore coronary flow as soon as possible by means of percutaneous intervention or pharmacological thrombolysis to salvage as much myocardium at risk as possible.\(^1\) However, despite the significant improvement in patients’ prognosis achieved by these reperfusion strategies, there are still a significant number of patients who die, develop severe heart failure, or are discharged with a poor left ventricular ejection fraction.\(^2\) In addition, the therapeutic window for infarct size reduction is considerably narrow,\(^3\) so only those patients receiving prompt and successful reperfusion therapy are expected to attain major gain in terms of myocardial salvage. Although substantial efforts are being made in order to reduce door-to-balloon and door-to-needle times,\(^4\) reperfusion will continue to arrive too late for a significant number of patients. In this context, the only other option is to implement treatments that, used in conjunction with reperfusion, could enhance the salvage and functional recovery of ischaemic myocardium by directly interfering with the mechanisms of ischaemia–reperfusion injury.

Investigation of the mechanisms that contribute to myocardial cell death during ischaemia and reperfusion has attracted the interest of cardiovascular investigators over the last three decades and several promising pharmacological targets have been identified. However, despite the fact that experimental studies using pharmacological tools against some of these targets have consistently shown that it is possible to reduce infarct size by interventions applied at the time of reperfusion, the development and testing of drugs for their clinical use has yet to be accomplished for most of these targets and has failed in others.\(^5\) The discovery of post-conditioning has re-awakened the interest in reperfusion injury among researchers and clinicians, since its efficacy in protecting the heart at the time of reperfusion not only represents another confirmation of the existence of lethal reperfusion injury but also demonstrates its clinical applicability. In addition, the role of the mitochondrial permeability transition pore (mPTP) as a central player in reperfusion injury has been recognized. The benefits of both post-conditioning and inhibition of mPTP have been supported by recently published proof-of-concept trials.\(^6,7\) However, the interest of pharmaceutical companies and research funding agencies in developing new drugs against reperfusion injury acting on other previously described pharmacological targets is still very limited, which results in the waste of extensive mechanistic knowledge acquired during the last few decades.

The present article will review pharmacological targets against reperfusion-induced cell death other than mPTP, in

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particular those related to loss of Ca\textsuperscript{2+} homeostasis that have not yet resulted in the development of therapies for patients with AMI.

1.1 Mechanisms of immediate irreversible reperfusion injury

Histological analysis has shown that post-reperfusion infarcts are almost exclusively formed by areas of contraction band necrosis composed of hypercontracted myocytes, in which the cell geometry is dramatically distorted as a consequence of extreme cell shortening and the sarcolemma is disrupted. This pattern appears within the first minutes after reperfusion and is critically determined by loss of Ca\textsuperscript{2+} homeostasis.\textsuperscript{10}

There is solid evidence that mechanical stress caused by excessive contractile activation, known as hypercontracture, critically contributes to sarcolemmal rupture and cell death.\textsuperscript{11} Excessive activation of the contractile machinery when ATP production recovers in the presence of altered Ca\textsuperscript{2+} handling is a fundamental mechanism of hypercontracture.\textsuperscript{12} Ca\textsuperscript{2+} overload in reperfused myocardium is largely due to its influx through reverse sarcolemmal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (NCX) during the preceding ischaemia and during the first minutes of reperfusion.\textsuperscript{13,14} Reverse-mode operation of NCX is the consequence of a decreased transsarcolemmal Na\textsuperscript{+} gradient, which in turn is caused by increased Na\textsuperscript{+} concentration associated with the mechanisms of intracellular pH (pHi) correction and lasts until restoration of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity normalizes it and membrane potential recovers. In cardiomycocytes with restored mitochondrial energy production, this excessive load of intracellular Ca\textsuperscript{2+} is shifted in an oscillatory manner between cytosol and sarcoplasmic reticulum (SR), resulting in the rapid repetition of high cytosolic peak concentrations of Ca\textsuperscript{2+}. These Ca\textsuperscript{2+} transient peaks cause the uncontrolled activation of the contractile machinery that results in hypercontracture.\textsuperscript{10,15}

The histological pattern of contraction band necrosis might also be due to a rigor-associated, Ca\textsuperscript{2+}-independent contracture.\textsuperscript{16} Rigor contracture occurs during ischaemia and could also occur after reperfusion when energy recovers slowly while cytosolic ATP is maintained at a sub-millimolar but non-zero concentration, as in re-oxygenated myocardial cells with heavily injured mitochondria.\textsuperscript{17} Massive mPTP opening at reperfusion could trigger rigor contracture, since it inhibits energy production. However, induction of mPTP opening with pulsed laser illumination of TMRE-loaded myocytes results in limited rigor shortening but not in the extreme cell shortening characteristic of hypercontracture.\textsuperscript{18}

The sarcolemmal tolerance to mechanical stress is critically reduced by increased cytoskeletal fragility\textsuperscript{19–21} due to calpain-dependent hydrolysis of proteins from the sarcolemma and cytoskeleton.\textsuperscript{22} In addition, calpain contributes to the delocalization and dysfunction of the sarcolemmal Na\textsuperscript{+}-pump by degrading ankyrin, the protein that attaches it to the fodrin cytoskeleton.\textsuperscript{23} Impaired normalization of the cytosolic Na\textsuperscript{+} concentration results in a further Ca\textsuperscript{2+} rise and calpain activation, closing a vicious circle.\textsuperscript{24} Finally, sarcolemmal rupture can propagate to adjacent reperfused cells that are already fragility and have Na\textsuperscript{+}-pump dysfunction through passage of Na\textsuperscript{+} via gap junctions\textsuperscript{25} and subsequent reverse Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange in adjacent cells.

1.2 Targeting reperfusion injury

Knowledge on the mechanisms of cell death during myocardial ischaemia/reperfusion has disclosed a number of potential therapeutic targets (Figure 1). Strategies aimed at preventing mPTP opening at reperfusion are reviewed elsewhere in this issue, and will not be discussed in the present article. A large number of studies propose that the activation of the pro-survival kinase cascade (RISK pathway) phosphorylates the glycogen synthase kinase 3\beta (GSK3\beta), preventing the opening of mPTP.\textsuperscript{26} However, it has recently been questioned whether the association of RISK activation with less infarction is a cause or consequence of cardioprotection.\textsuperscript{27}

A large amount of data demonstrates that Ca\textsuperscript{2+} plays a central role in the processes leading to reperfusion-induced cell death, and many experimental studies have identified potential therapeutic targets related to Ca\textsuperscript{2+} overload during reperfusion and its consequences. Although not reviewed here, Ca\textsuperscript{2+} is the first identified and better characterized mPTP opener. Ca\textsuperscript{2+} concentrations in the range of 50–200 \(\mu\)M directly induce mPTP opening in respiring mitochondrial preparations; however, the role of Ca\textsuperscript{2+} overload as a determinant of mPTP opening during reperfusion appears to be less prominent and other facts such as oxidative stress are very important.\textsuperscript{28,29}

2. Targeting Ca\textsuperscript{2+} overload

Ca\textsuperscript{2+} is a second messenger in numerous signalling pathways and regulates many processes including muscle contraction and cardiac rhythm. It is therefore theoretically evident that the loss of Ca\textsuperscript{2+} homeostasis during ischaemia/reperfusion may be detrimental. Although intracellular [Ca\textsuperscript{2+}]\textsubscript{i} starts to rise during ischaemia,\textsuperscript{30} it has been demonstrated that an additional Ca\textsuperscript{2+} entry occurs during the first
minutes of reperfusion and that its prevention reduces infarct size in different species and models. The most investigated and promising targets directed at preventing Ca\(^{2+}\) entry during reperfusion are discussed below.

### 2.1 Na\(^{+}\)/H\(^{+}\)-exchanger

Experimental studies have demonstrated marked infarct size reduction with a high degree of conformity between research groups, species and models when inhibitors of Na\(^{+}\)/H\(^{+}\)-exchanger (NHE) are administered prior to ischemia. In contrast, the efficacy of NHE inhibitors administered just prior to reperfusion remains controversial. In the ESCAMI trial, the NHE inhibitor eniporide given before reperfusion therapy in patients with ST-elevation AMI did not limit infarct size or improve clinical outcome. In contrast, when the NHE inhibitor cariporide was administered before coronary artery bypass graft surgery, a reduction in perioperative infarction was observed in a subgroup of the GUIDANT trial and in the recent EXPEDITION trial. However, treatment with cariporide was associated with higher rates of short-term mortality and cerebrovascular events in this latter trial, precluding its clinical use.

These divergent results of the clinical trials with NHE inhibitors, which depend on the timing of their administration, are consistent with the experimental data showing that these drugs protect mainly by delaying the progression of myocardial injury during ischemia. However, NHE also results in a net pH-dependent Na\(^{+}\) influx during the first minutes of reperfusion, which is coupled to Ca\(^{2+}\) influx through reverse-mode NCX correction. The relative contribution of NHE to Ca\(^{2+}\) entry during reperfusion and, therefore, to reperfusion injury is dependent on the ability of cardiomyocytes to restore Na\(^{+}\)/K\(^{-}\)-ATPase activity. There is evidence suggesting that the role of NHE at reperfusion is underestimated due to the compensatory action of bicarbonate transporters. Inhibition of NHE results in only a small effect on kinetics of Na\(^{+}\) and pH recovery during reperfusion. In contrast, simultaneous inhibition of both NHE and Na\(^{+}\)/HCO\(_3\) cotransport has been shown to delay recovery of pH and to attenuate development of hypercontracture and reperfusion-induced cell death. These results could help explain the lack of consistency of the studies aimed at inhibiting NHE during reperfusion and, in addition, support the benefits of delaying pH recovery.

Finally, all the NHE inhibitors that have been clinically evaluated inhibit NHE activity by competing with Na\(^{+}\), and their systemic administration could inhibit NHE elsewhere in the body and trigger adverse non-cardiac effects such as those observed in the EXPEDITION trial. Recently, an alternative strategy to achieve the therapeutic benefits of reduced cardiac NHE activity has been suggested. This approach is based on targeting the molecular signalling pathways that stimulate cardiac NHE activity under pathological conditions such as one involving 90 kDa ribosomal S6 kinase (P90RSK). Whether drugs against RSK could prevent reperfusion injury remains to be determined.

### 2.2 Na\(^{+}\)/Ca\(^{2+}\)-exchanger

Na\(^{+}\)/Ca\(^{2+}\)-exchanger inhibitors applied at the time of reflow have been shown to reduce experimental myocardial infarction, confirming that the first minutes of reperfusion result in an additional Ca\(^{2+}\) influx through the reverse mode of NCX coupled to normalization of pH and Na\(^{+}\)/K\(^{-}\). However, no NCX inhibitor has been developed for use in humans. Under normal conditions, activity of NCX during diastole in its forward mode contributes to Ca\(^{2+}\) extrusion and maintenance of Ca\(^{2+}\) homeostasis, whereas the reverse mode of NCX during action potential depolarization contributes to the systolic Ca\(^{2+}\) transient in a species-dependent manner. Inhibition of NCX has no detrimental effect in normal cardiomyocytes, and genetic ablation of NCX1 in mice does not alter cardiac function. However, the situation is different during reperfusion. In cardiomyocytes with severe Na\(^{+}\) overload and reduced membrane potential, NCX operates in the reverse mode and its inhibition attenuates Ca\(^{2+}\) overload and limits reperfusion injury. However, in cardiomyocytes in which recovery of Na\(^{+}\)/pump activity allows correction of Na\(^{+}\) overload, the forward mode of NCX contributes to Ca\(^{2+}\) extrusion. Therefore, drugs inhibiting this mode are expected to impair cytosolic Ca\(^{2+}\) normalization and be detrimental. This has been demonstrated in a previous study in which, while inhibition of NCX with KB-R7943 markedly reduced infarct size when given during the first 4 min of reperfusion, this drug was detrimental when treatment was prolonged to the first 15 min of reperfusion.

A better alternative would be the development of new inhibitors specifically targeting reverse-mode exchange. During the last few years, new benzyloxyphenyl NCX inhibitors more potent and selective than KB-R7943 have been developed (SEA0400, SN-6). These molecules tend to block the reverse mode more effectively than the forward mode under unidirectional ionic conditions. However, this apparent preference for the forward mode disappears under conditions allowing alternating net forward and reverse NCX operation during the cardiac cycle. Under these conditions, inhibition of NCX impairs Ca\(^{2+}\) regulation. A safe translation of NCX inhibition to the clinical setting is therefore dependent on the identification of a time window during which NCX exclusively operates in its reverse mode. Such a narrow time window would require intracoronary infusion.

Caldaret (MCC-135, 5-methyl-2-(1-piperazinyl) benzene-sulfonic acid monohydrate) was shown to limit infarct size in models involving transient coronary occlusion. This effect was associated with attenuated Ca\(^{2+}\) overload attributable to inhibition of Ca\(^{2+}\) influx via NCX and enhanced SR Ca\(^{2+}\) uptake, although the direct involvement of these mechanisms has not been documented. Recently, the cardioprotective effects of caldaret have been tested in patients with STEMI. In the CASTEMI study, patients with a large STEMI were allocated to receive a 48 h infusion of caldaret or placebo starting before primary percutaneous coronary intervention. The drug did not appear to reduce infarct...
size in the target population of patients with pre-procedural coronary occlusion, although trends to a favourable effect were observed in the subset with anterior infarctions.45 A subanalysis of this population revealed a significant decrease in the incidence of severe LV dysfunction in patients receiving caldaret.46 However, in another trial, caldaret failed to reduce infarct size or preserve LV ejection fraction.

2.3 Sarcoplasmic reticulum

The SR plays a critical role in Ca\(^{2+}\) handling during the first minutes of reperfusion48 and has been proposed as a primary target for protection during this period. SR serves as an intracellular sink for excess cytosolic Ca\(^{2+}\), but when its capacity is exceeded it results in repetitive uptake and release of Ca\(^{2+}\) oscillations that propagate across the cell length as Ca\(^{2+}\) waves by a Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism that contributes to lethal reperfusion arrhythmias.49 In vitro, it is possible to protect re-oxygenated myocardial cells from Ca\(^{2+}\)-dependent hypercontracture by blocking these oscillatory movements of Ca\(^{2+}\) between the SR and cytosol.50 Moreover, since the SR and mitochondria are coupled in a functional unit in intact cells,51 it can also be expected that Ca\(^{2+}\) oscillations may contribute to mPTP opening, although this has not yet been demonstrated.

Several studies suggest a role of SR Ca\(^{2+}\)-ATPase (SERCA) activity in reperfusion injury, although the nature of this role is a matter of debate. Increased activation of SERCA augments SR Ca\(^{2+}\) load, which may lead to increased oscillatory Ca\(^{2+}\) release and worsening of reperfusion injury.52 On the other hand, SERCA activity may favour cytosolic Ca\(^{2+}\) removal, prevent propagation of Ca\(^{2+}\)-induced Ca\(^{2+}\) release waves, and extinguish Ca\(^{2+}\) oscillations.49 Overexpression of SERCA reduced infarct size and preserved cardiac function in rats subjected to transient LAD ligation and, as mentioned earlier, the cardioprotective effects of caldaret are suggested to be mediated, at least in part, by its effect on SERCA.44 Conversely, heterozygous SERCA knockout mice resulted in increased cytosolic Ca\(^{2+}\) concentration, reduced functional recovery, and larger infarcts after transient ischaemia.54 Accordingly, enhancement of SERCA activity has been proposed as potential therapeutic strategy against reperfusion injury. Phosphorylation of phospholamban, a regulator of SERCA activity, by cAMP or cGMP-dependent protein kinase (PKA or PKG) at Ser16 or Ca\(^{2+}\)-calmodulin-dependent kinase (CaMK) at Thr17 increase SR Ca\(^{2+}\) activity,55,56 while prolongation of contractile inhibition from Ca\(^{2+}\) depletion, either by Ca\(^{2+}\)-induced Ca\(^{2+}\) release or by blockers of contractility, such as BDM, has proven to be feasible in pigs and could theoretically be used in patients receiving primary percutaneous coronary revascularization, the potential severe effect of involuntary administration to contractile myocardium is an important limitation for its clinical use. BDM is effective when used at high concentrations, and its phosphatase activity also suppresses contraction of skeletal and smooth muscles and has effects on different ion channels.

Apart from BDM, the cardioprotective effects of other strategies discussed below, such as acidosis and stimulation of the cGMP/PKG pathway, can be also attributed in part to their effects on contractility and prevention of hypercontracture, as they reduce the Ca\(^{2+}\) sensitivity of myofibrils.

3. Targeting mechanisms activated by Ca\(^{2+}\) overload

A major limitation of strategies aimed to limit Ca\(^{2+}\) overload at reperfusion is that they cannot prevent its accumulation during prior ischaemia. An alternative strategy is to develop drugs directed to inhibit those mechanisms activated by Ca\(^{2+}\) overload during reperfusion that have been demonstrated to contribute to cell death.

3.1 Hypercontracture

Several lines of evidence support the importance of the mechanical stress caused by hypercontracture as the most prominent pathomechanism of cardiomyocyte death during reperfusion: (i) the occurrence of hypercontracture during the first minutes of reperfusion has been documented in vitro by microscopy techniques, and in vivo by intramyocardial ultrasonometry,9,13,19,20 (ii) there is a tight correlation between the magnitude and time course of hypercontracture, enzyme release, and contraction band necrosis,9,13 (iii) those strategies aimed at transiently inhibiting contractility at the onset of reperfusion result in transient inhibition of enzyme release for the same period of time,24,57 while prolongation of contractile inhibition until cardiomyocytes recover Ca\(^{2+}\) control results in very pronounced infarct limitation in a variety of models57–59 (Figure 2).

Experimentally, various strategies have been shown to interfere with Ca\(^{2+}\) overload-induced hypercontracture. The drug more extensively used to inhibit the myofilamentary machinery is 2,3-butanedione monoxime (BDM), a reversible blocker of actomyosin ATPase. Administration of BDM during the early phase of reperfusion markedly reduces the infarct size and enhances post-ischaemic recovery of contractile function.57,59 BDM, as any contractile blocker, has to be administered selectively into the area at risk since otherwise it would produce cardiac arrest. Although this strategy has proven to be feasible in pigs and could theoretically be used in patients receiving primary percutaneous coronary revascularization, the potential severe effect of involuntary administration to contractile myocardium is an important limitation for its clinical use. BDM is effective when used at high concentrations, and its phosphatase activity also suppresses contraction of skeletal and smooth muscles and has effects on different ion channels.

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3.2 Calpains

Calpains are a family of non-lysosomal Ca\(^{2+}\)-dependent thiol proteases implicated in a wide range of basic cellular functions, including apoptosis, proliferation, and cell migration. Under pathologic conditions in which Ca\(^{2+}\) homeostasis is lost, as occurs during ischaemia–reperfusion, the tight control of the calpain system is disturbed, causing inappropriate activation.60 Calpain hydrolysis of known calpain substrates and the use of calpain inhibitors in different models of ischaemia–reperfusion have consistently demonstrated that calpain activation occurs prior to membrane damage and plays an important role in reperfusion-induced contractile dysfunction and cell death due to the proteolysis of a wide variety of proteins.22,24,61 Several mechanisms have been proposed by which calpain may contribute to reperfusion injury (Figure 3). During
reperfusion calpains hydrolyse proteins from the sarcolemmma and the cytoskeleton including α-fodrin and ankyrin. Alpha fodrin forms the backbone of the membrane cytoskeleton. Its degradation correlates with increased fragility of the membrane, reducing the tolerance of the sarcolemma to acute cell swelling and contractile activation during reperfusion.22,62 Ankyrin has a central domain that binds to α-fodrin and an N-terminal domain that interacts with several receptors and channels, including the α subunit of Na⁺/K⁺-ATPase.63 Binding to ankyrin connects the Na⁺/K⁺-ATPase to the fodrin-based membrane cytoskeleton and determines its specific localization in the membrane and its correct function.23 During reperfusion, calpain degradation of both fodrin and ankyrin not only causes sarcolemmal fragility but also detachment of the Na⁺-pump from its anchorage to the fodrin-based membrane skeleton, inducing dysfunction of the sarcolemmal Na⁺-pump, which results in impaired normalization of cytosolic Na⁺ concentration and in further Ca²⁺ influx via reverse Na⁺/Ca²⁺ exchanger.24 In addition, activated calpain, by cleaving Bid into an active form, has been described to induce the release of cytochrome c and other proapoptotic factors.61

The kinetics of calpain activation during ischaemia-reperfusion is poorly defined. Acidosis inhibits calpain activity in vitro.64 Different groups including ours have demonstrated that the effectiveness of post-conditioning in limiting infarct size depends on its ability to delay pHi recovery during reperfusion.65,66 Moreover, we have recently shown that prolongation of intracellular acidosis during reperfusion by either post-conditioning or by acidic perfusion limits myocardial necrosis and improves the contractile recovery at least in part through attenuation of calpain activation.65 These data support the idea that calpain activation occurs during reperfusion and that, therefore, a window of opportunity exists for preventing its activation. Recently, it was reported that the administration of a calpain inhibitor during reperfusion reduced

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**Figure 2** Data showing that hypercontracture play a determinant role in reperfusion induced cell death. (A) Representative traces of left ventricular pressure (LVP) and (B) time course of LDH release during the first 30 min of reperfusion in rat hearts subjected to ischaemia-reperfusion and perfused or not during the first 5 min of reperfusion with butanedione monoxime (BDM). (C) Drawing of myocardial sections stained with Masson’s trichrome showing in black the area of contraction band necrosis and (D) area at risk and infarct size corresponding to pig hearts subjected to transient LAD ligation with and without intracoronary infusion of BDM during the first 30 min of reperfusion, *P<0.05 (modified from24,57).
However, gap junction inhibitors have important limitations that preclude their translation to clinics. One is the lack of specific, rapid, and reversible inhibitors. In addition, all these drugs have dramatic electromechanical effects incompatible with the normal function of non-ischaemic myocardium. Even if drugs are intracoronarily delivered into the area at risk, there is an increased risk of arrhythmias during regional contractile blockade. Application of this knowledge to development of more adequate inhibitors of gap junctions will offer the possibility to evaluate the balance between the potential risk, mainly arrhythmogenesis, and the benefits of infarct limitation in patients undergoing primary percutaneous intracoronary interventions.

5. Therapies directed at multiple targets

Some protective strategies that have been proposed interfere simultaneously with several mechanisms of reperfusion injury, which make them particularly interesting.

5.1 Acidic reperfusion

Studies in several experimental models have demonstrated that a delay in pHi recovery induced by pharmacological treatments or transient reperfusion with either respiratory or metabolic acidosis could be protective. Furthermore, it has been shown that the optimal protection is obtained when the prolongation of extracellular acidosis is circumscribed to the first 2–3 min of reperfusion. The potential value of strategies based on interfering with the rapid normalization of pHi has been reinforced with recent studies showing that post-conditioning protection depends on prolongation of acidosis.

Prolongation of acidosis during the first minutes of reperfusion may protect cardiomyocytes by multiple mechanisms, although their relative importance has not been established. Intracellular acidosis inhibits myofibrillar contractility, preventing Ca$^{2+}$-dependent hypercontracture during initial reperfusion and calpain activity, attenuating Ca$^{2+}$-dependent proteolytic injury occurring during reperfusion. Low pHi also inhibits mPTP opening and explains why mPTP remains closed during ischaemia. Recently, it was proposed that transient acidosis leads to the activation of pathways involving Akt and extracellular signal-regulated kinases, leading to the prevention of mPTP opening once the pHi is normalized. In addition, several studies propose that acidosis could prevent Ca$^{2+}$ entry by inhibition of NCX. Finally, acidosis maintains gap-junctions in a closed state, impairing the propagation of hypercontracture.

Despite the huge amount of experimental data supporting that prolongation of acidosis during initial reperfusion is a promising option to limit infarct size, only few studies have analysed the effect of transient acidosis during reperfusion in models close to the clinical setting, and the optimal conditions of its application (duration, target pH value, and type of acidic solution) are far from being defined. This point is essential for translation to patients since the efficacy of this strategy critically depends on the level of pHi and the duration of acidosis.
5.2 The cGMP/PKG pathway

The cGMP/PKG pathway is severely depressed in cardiomyocytes and endothelial cells after prolonged ischaemia.\(^{78,79}\) Preservation of this pathway with sodium nitroprusside, natriuretic peptides, and cGMP analogues has been demonstrated to protect against reperfusion injury.\(^{56,80,81}\) More recently, the use of specific inhibitors of phosphodiesterases has added further support to the concept that up-regulation of the cGMP-PKG pathway can effectively reduce ischaemia-reperfusion injury.\(^{82}\)

PKG could modulate several targets. As noted above, a prominent one is its effect on phospholamban, enhancing SERCA activity, accelerating normalization of cytosolic Ca\(^{2+}\) concentration and reducing development of hypercontracture.\(^{56}\) The cardioprotective actions of cGMP have also been related to the PKG-dependent effects on the sensitivity of myofibrils to Ca\(^{2+}\).\(^{83,84}\) This effect could be the consequence of a direct phosphorylation of troponin I and/or related to direct or indirect effects of PKG on myosin light chain phosphatase.\(^{83,85}\) Finally, one study showed that an mK\(_{ATP}\) channel opens in isolated mitochondria incubated with cGMP and PKG.\(^{86}\) However, although this study suggested a PKG-dependent phosphorylation site in the mitochondrial outer membrane, its link with mK\(_{ATP}\) remains unknown. Recently, PKG-dependent mK\(_{ATP}\) opening has been proposed to mediate, at least in part, the cardioprotective effects of nicorandil.\(^{87}\) Intravenous administration of nicorandil, which is a hybrid of a K\(_{ATP}\) channel opener and a nitrate, in conjugation with reperfusion therapy in patients with AMI improved myocardial viability and functional outcome in some studies.\(^{88,89}\) The more recent and larger J-WIND trial analysed the effects of intravenous nicorandil and human atrial natriuretic peptide on infarct size and cardiovascular outcome in AMI patients receiving reperfusion therapy.\(^{90}\) Whereas nicorandil was not protective, human atrial natriuretic peptide appeared to reduce infarct size, estimated by total creatine kinase release, and increased left ventricular ejection fraction.\(^{90}\) Although the exact mechanism of protection afforded by cGMP remains unclear, studies testing this pathway are the exceptions to the predominantly negative results obtained in trials aimed to reduce reperfusion injury.\(^{90,91}\) and represent an example of successful translation of basic research knowledge to patients. Large-scale trials to target outcomes such as cardiovascular death are now necessary to confirm the value of this therapy.

5.3 Adenosine

Adenosine has extensively been evaluated in relation to reperfusion injury. The use of agonists and antagonists of adenosine receptors has demonstrated the ability of adenosine to reproduce the cardioprotective effects of ischaemic pre-conditioning.\(^{92,93}\) However, the effect of adenosine administered at the onset of reperfusion is less consistent among studies.\(^{94,95}\) The cardioprotective effects of adenosine have been related to its vasodilatory, anti-thrombotic, anti-inflammatory, and antigenic properties, and more recently to activation of the RISK pathway.\(^{26}\) It has been proposed that the activation of PI3K/Akt signal transduction induces the stimulation of soluble guanylate cyclase that in turn results in PKG activation,\(^{96}\) suggesting that adenosine, through a PKG-dependent phosphorylation of phospholamban, could also be cardioprotective by modulating Ca\(^{2+}\) handling.

Different clinical trials have assessed the ability of adenosine to limit infarct size in patients with AMI. In the small, open-label AMISTAD trial, this drug appeared to reduce infarct size when given as an adjunct to thrombolytic therapy in patients with anterior infarctions.\(^{97}\) However, in the larger AMISTAD-2 trial, intravenous adenosine (50 or 70 µg/kg/min for 3 h starting within 15 min of reperfusion therapy) failed to reduce the occurrence of new congestive heart failure or death in patients with anterior AMI receiving thrombolysis or primary angioplasty.\(^{98}\) A significant reduction in infarct size was observed in a subgroup of these patients studied by 99mTc scintigraphy receiving the highest dose of the drug.\(^{99}\) Although in another small study, intracoronary adenosine infusion at the time of mechanical reperfusion was associated with less creatine kinase release, more preserved coronary flow, improved ventricular function, and a more favourable clinical course,\(^{100}\) the adenosine analog AMP579 failed to reduce infarct size in the ADMIRe trial.\(^{101}\) Unfortunately, in this trial AMP579 was not infused until the opening of the artery was confirmed, ignoring the experimental evidence that indicates that drugs given at reperfusion must be present from the first minute. The overall conclusion obtained from these studies is the lack of definitive evidence that adenosine or selective agonists are protective at reperfusion.

5.4 Other drugs

During the last few years, drugs and non-drug approaches (such as hypothermia) aimed at other mechanisms of injury have been tested as adjuncts of reperfusion therapy in AMI patients. Although it will not be discussed in the present review, glucose–insulin–potassium (GIK) deserves a brief comment due to the great expectations that it raised. GIK has been shown to reduce infarct size in the majority of experimental studies by different mechanisms including activation of Akt and p70s6 kinase cell-survival signalling.\(^{102}\) However, the clinical benefit of GIK infusion in patients with STEMI is unclear and, while some smaller trials suggest a benefit in specific subgroups, in two large, controlled trials, GIK infusion provided no benefit.\(^{102}\) Late administration, hyperglycaemia and hyperkalaemia have been suggested to explain these negative results. The immediate trial will assess the potential effect of GIK when these facts are precluded.

Erythropoietin has also aroused great interest. Numerous in vivo studies have shown a protective effect against reperfusion injury even when erythropoietin is administered at the onset of reperfusion.\(^{103}\) This cardioprotection has been associated to the activation of PI3K/Akt and MAPK pathways.\(^{104}\) However, the only pilot trial that has analysed the effect of an erythropoietin analog in AMI patients showed no significant differences in ejection fraction.\(^{105}\)

6. Concluding remarks: closing the gap between targets and drugs

Although experimental evidence of the existence of lethal reperfusion injury is extremely solid, its relative contribution to final infarct size in patients with acute coronary syndrome has not yet been adequately characterized. It is
tempting to speculate that the contribution of reperfusion injury to final infarct size depends on conditions like the duration of ischaemia. Extrapolating the results obtained in most animal studies to patients receiving reperfusion after 10 h of coronary occlusion is far from safe, and it would not be surprising that lethal reperfusion injury would be irrelevant in these patients. But a significant fraction of patients receive reperfusion within few hours after pain onset, and there are no solid reasons to believe that reperfusion injury in their hearts would be essentially different from that observed in other animal species. In any case, the only way to definitively establish the role of lethal reperfusion injury in patients is through controlled, randomized, blind studies in which infarct size and area at risk are accurately determined. Initial evidence suggests that could be the case.6,7,90

In an era when shortage of new pharmacological targets is considered a major problem in health science, particularly in the cardiovascular area, the existence of targets for which no drugs have been developed may appear surprising, more so if we consider the enormous social impact of myocardial infarction. The explanations for this phenomenon are multiple.106 The priority of the objective of delivering timely reperfusion to patients in whom it is indicated, the limited economic interest of coadjuvant therapies to be used as a single dose, possibly once in a patient’s life, have been important factors. Globalization and increasing economic pressure work against new drug development programs. This results in testing of available drugs developed for indications other than reperfusion injury, rather than in the expensive and risky identification and testing of new molecules. It is the authors’ opinion that breakthroughs in mechanistic insight will probably require the use of a new integration approach that can result in quantitative pathways and network models of cardioprotection signalling through the use of systems biology techniques. However, a more predictive mechanistic understanding of reperfusion injury will not lead to better treatments and outcomes for patients with AMI without a successful program of innovation. This will require a close cooperation of investigators and industry and the support of non-profit biomedical research institutions and funding agencies at national and international levels. These efforts should result in the discovery of new drugs for reperfusion injury suitable for clinical use whose effectiveness would be based on the improvement of cellular Ca2+ handling and attenuation of the consequences of Ca2+/ overload.

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