Inhibition of mitochondrial permeability transition pore opening: translation to patients

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1. Myocardial lethal reperfusion injury

Coronary heart disease is the leading cause of death in western countries. Myocardial infarction is a disabling disease, with infarct size being a major determinant of mortality after this common acute coronary event. The limitation of infarct size has therefore been an important target for therapeutic strategies to improve patient outcomes. Currently, the most effective way to limit infarct size is to reperfuse the jeopardized myocardium as soon as possible with the use of coronary angioplasty or thrombolysis and to prevent re-occlusion of the coronary artery with an efficient antiplatelet and antithrombotic therapy. The development of thrombolysis and coronary angioplasty has clearly improved the prognosis of patients with acute myocardial infarction (AMI). Numerous studies have demonstrated that reperfusion limits infarct size and improves functional recovery.1–3

Despite these beneficial effects, Jennings et al. first suggested that reperfusion might also cause some functional as well as structural damages to the heart.4 Braunwald and Kloner termed reperfusion ‘a double-edged sword’ since it caused myocardial stunning, ventricular arrhythmias, and no-reflow.5–10

Besides these functional alterations, it has been shown that reperfusion caused irreversible damage to the previously ischaemic myocardium.11 There has been much debate as to whether reperfusion would simply accelerate cell death or truly kill cardiomyocytes that would otherwise have survived (in the absence of the deleterious effects of reperfusion). Although several investigations during the past decades reported that pharmacological interventions at reperfusion might attenuate cardiomyocyte cell death, the concept of lethal reperfusion injury faced some skepticism mainly due to contradictory results, inconsistent data, and failure to reproduce these data in in vivo models and in all animal species.12–15

The description of postconditioning by Vinten-Johansen’s group, together with numerous other studies using pharmacological agents that reduced infarct size when given at reperfusion, established the existence of lethal reperfusion injury.16,17 These authors first reported that brief episodes of ischaemia performed just at the onset of reperfusion following a prolonged ischaemic insult dramatically reduced infarct size. This observation has been confirmed in several experimental preparations and animal species.18–21 These well-designed, controlled studies performed in independent laboratories have demonstrated the existence of lethal reperfusion injury, a complex process in which cellular necrosis and apoptosis were both involved.22,23
1.1 Mitochondrial permeability transition in lethal reperfusion injury

Lethal reperfusion injury of the myocardium is a complex phenomenon that encompasses several aetiologies. Most of the detrimental effects of reperfusion are triggered within the first minutes following the re-opening of the occluded coronary artery. However, most of the cellular disturbances that occur at the time of reperfusion are determined or critically dependent on ischaemia-induced abnormalities. During ischaemia, the increase of anaerobic glycolysis results in a progressive accumulation of protons and lactic acid, eventually inhibiting glycolytic flux and synthesis of ATP. The cardiomyocyte attempts to correct acidosis via the Na\(^+\)/H\(^+\) exchanger and will consequently load with Na\(^+\). This Na\(^+\) excess cannot be extruded from the cytosol because of the Na\(^+\)/K\(^+\)-ATPase failure due to the lack of ATP. Secondary activation of the Na\(^+\)/Ca\(^{2+}\) exchanger, in its reverse mode, will help pump Na\(^+\) out of the cell, but induces cytosolic accumulation of Ca\(^{2+}\). Prolonged ischaemia induces progressive failure of the ionic homeostasis, which ultimately causes accumulation of intracellular Na\(^+\) and Ca\(^{2+}\), ATP decline, and development of ischaemic contracture.

In the first minutes of reperfusion, the rapid correction of acidosis via the Na\(^+\)/H\(^+\) exchanger, the Na/HCO\(_3\) symporter, and the washout of lactate will cause secondary activation of the Na\(^+\)/Ca\(^{2+}\) exchanger in the reverse mode and aggravate cytosolic Ca\(^{2+}\) accumulation. The abrupt re-exposure of the ischaemia-inhibited mitochondrial respiratory chain to oxygen will generate a membrane potential to drive ATP synthesis, but then lead to rapid matrix Ca\(^{2+}\) overload and massive production of oxygen-derived free radicals. These two factors are the major triggers of the opening of the mitochondrial permeability transition pore (mPTP).

Under normal physiological conditions, the mitochondrial inner membrane is impermeable to almost all metabolites and ions, and the mPTP is in a closed conformation. Under stress conditions, the mPTP opens and allows the equilibration of molecules <1500 da. The osmotic force of matrix proteins results in matrix swelling, leading to further rupture of the mitochondrial outer membrane and release in the cytosol of pro-apoptotic factors like cytochrome c. In addition, the disruption of mitochondrial membrane potential also results in the ATP synthase to behave as an ATPase and accelerates energy depletion secondary to the ischaemic insult. In the isolated heart model, Di Lisa et al. demonstrated that the cytosolic release of NAD\(^+\), a surrogate marker of mPTP opening, occurs at the time of reperfusion following a prolonged ischaemic insult. Griffiths et al. used the \(^{[3}H\)2-deoxyglucose entrapment technique to investigate the kinetics of in situ mPTP opening and demonstrated that mPTP opening does not happen during ischaemia, but occurs within the first 5 min of reflow following ischaemia in the isolated rat heart. Importantly, the time course of mPTP opening appeared to match the rapid correction of pH that occurs at reperfusion (Figure 1A). Recent in vivo studies support this concept by showing that postconditioning mediates its cardioprotective effects via prolonged transient acidosis during the early reperfusion phase (Figure 1B).

1.2 Inhibition of cyclophilin D as a target to attenuate lethal reperfusion injury

Additional evidence for a major role of the mPTP in lethal reperfusion injury recently came from the use of transgenic mice lacking cyclophilin D (cypD). CypD, which is recognized as a key molecular component of the mPTP, is a mitochondrial member of the family of peptidyl-prolyl cis-trans isomerases. Although still debated, it has been reported that, in the presence of high matrix Ca\(^{2+}\) concentration, cypD may modify the conformation of the ANT, so that it no longer functions as a nucleotide transporter, but rather as a channel component for the mPTP. The molecular structure of the mPTP remains poorly known and might involve, besides cyclophilinD, various proteins including VDAC or ANT. Unfortunately, their precise role is still elusive and no pharmacological agent targeting these proteins is currently available for clinical trials. Cyclosporine A (CsA) is considered to inhibit mPTP opening by preventing the binding of cypD to the ANT. In vivo, cypD-deficient mice have been shown to develop smaller...
infarcts following a prolonged coronary artery occlusion followed by reperfusion. Recently, Lim et al. reported that cypD-deficient mice cannot be postconditioned, further suggesting that lethal reperfusion injury is mediated by mPTP opening. These results strongly support that the mPTP opening, triggered by mitochondrial Ca\(^2\+) overload and overproduction of reactive oxygen species, plays a central role in lethal reperfusion injury.

The importance of the mPTP in the ischaemia-reperfusion-induced necrotic death of various organs including the heart, brain, and liver was initially recognized through the use of mPTP inhibitors such as CsA and sanglifehrin A (SfA). Hausenloy et al. first reported that CsA or SfA, given at the time of reperfusion, limited infarct size in the isolated rat heart. Argaud et al. demonstrated that the specific mPTP inhibitor Nim811, a cyclosporine derivative, both increased the resistance of mPTP to Ca\(^2\+) overload and limited infarct size when given at the time of reflow. Interestingly, Argaud et al. reported that mitochondria isolated from the risk region of postconditioned hearts developed an enhanced resistance of the mPTP to Ca\(^2\+) overload. This pattern of inhibition of mPTP opening by postconditioning was very similar to that observed in hearts treated with the mPTP inhibitor Nim811 at the onset of reperfusion, as well as that of preconditioned rabbits. These studies suggest that ischaemic postconditioning may attenuate lethal reperfusion injury via the inhibition of mPTP opening.

2. Translation to patients

Unlike preconditioning, ischaemic postconditioning immediately appeared to clinicians as an unmet opportunity to determine whether lethal reperfusion injury existed in the human heart, and whether it could represent a new therapeutic target. Between 2004 and 2007, we performed three small-size phase II clinical trials aimed at demonstrating that: (i) ischaemic postconditioning could reduce infarct size and improve myocardial functional recovery several months after percutaneous coronary intervention (PCI), and (ii) pharmacological inhibition of mPTP opening by the commercially available mPTP inhibitor CsA could represent a pharmacological alternative to ischaemic postconditioning in AMI patients.

We demonstrated that PCI postconditioning was able to reduce infarct size by 30–40% and that this protection was persistent over time and resulted in a significant improvement of contractile function at 1 year after infarction. More recently, we reported that CsA significantly attenuated infarct size measured by cardiac enzyme release during the first 3 days of reperfusion and by magnetic resonance imaging (MRI) at day five after reflow. Several issues had to be addressed in order to reach these goals, including: (i) the safety and feasibility of these two types of interventions, (ii) the choice of an appropriate ‘human model’, and (iii) the choice of an adequate experimental design. We will briefly review here how we designed these small-size proof-of-concept trials.

2.1 Safety and feasibility of ischaemic postconditioning and cyclosporine A

Ischaemic postconditioning in AMI patients consisted of four cycles of 1 min inflation/1 min deflation of the angioplasty balloon in order to create repeated bouts of ischaemia and reperfusion just after re-opening of the culprit coronary artery. Repetition of balloon inflations and deflations is performed on a daily basis in all PCs performed for stable coronary artery lesions; in this regard, our experimental protocol did not add anything to the conventional PCI practice. The angioplasty balloon was inflated at low (2–4 atm) pressure, immediately upstream of the coronary stent, in order to avoid any damaging stress on the vessel wall or to the stent, and limit potential mobilization of remaining micro-thrombi. As a matter of fact, we did not notice any clinical complication or any evidence of coronary artery dissection, stent damage, or thrombosis in the limited number of patients who underwent PCI postconditioning. These findings are in agreement with Darling et al., 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. Experimental reports indicate that the amount of infarct size reduction by postconditioning may depend on the number, duration, and timing of the algorithm of brief cycles of ischaemia and reperfusion. We did not have the opportunity to test whether different types of algorithm would be more efficient in clinical practice; further studies are required to address this issue.

CsA has been used for many years as an immunosuppressive agent, a property related to its binding to the cytosolic calcium-activated protein phosphatase cyclophilin A. Long-term use of cyclosporine has several potentially detrimental effects, including renal and hepatic toxicity and increased susceptibility to infections and cancers. Following acute administration, anaphylactic reactions have been reported, as well as acute hypertension. In cardiac transplant patients, CsA may be used intravenously in order to prevent or treat acute graft rejection, with a daily dose up to 5 mg/kg. In our study, we used the commercially available form of CsA, Sandimmun® (Novartis). We chose the dose of 2.5 mg/kg because we had observed in in vivo animal models that this dose of CsA was able to reduce infarct size and did not cause any significant haemodynamic effect. Indeed, we did not observe any detectable clinical, haemodynamic, or biological sign of toxicity of CsA. In our clinical trial, CsA was administered as a bolus in an antecubital vein, within 10 min before re-opening of the occluded coronary artery. Measurement of blood concentration of CsA revealed that circulating levels were above values recommended in transplanted patients as soon as 1 min after reflow and for at least 3 h after reperfusion.

2.2 A human model to study interventions aimed at reducing lethal reperfusion injury

The primary objective of our proof-of-concept studies was to determine whether postconditioning or CsA administered at the time of reperfusion was able to limit infarct size. In order to demonstrate that a given therapeutic intervention reduces infarct size, one must be able: (i) to measure infarct size, (ii) to control the determinants of infarct size, and (iii) to control the conditions of reperfusion.

2.3 Measuring infarct size in acute myocardial infarction patients

Several techniques have been used to measure infarct size in clinical trials, including cardiac enzymes release,
single photon emission computed tomography (SPECT) imaging, or more recently MRI. We used the area under the curve of creatine kinase (CK) release over the first 3 days of reperfusion as the primary endpoint. Cardiac enzyme release has been well validated as a marker of infarct size. In our second PCI postconditioning study, SPECT imaging at 6 months after AMI confirmed persistent infarct size reduction as measured acutely by CK release. In the CsA study, MRI performed at day 5 after AMI in a subset of patients was in good agreement with CK release for the assessment of infarct size. It is interesting to notice that the amount of myocardial salvage observed in the two PCI postconditioning studies was very similar to that seen in the CsA study, i.e. comprised between 30 and 40% of the total infarct size measured in the control group. Furthermore, this infarct size reduction was comparable to that seen in most animal studies (Figure 2). This suggests that reperfusion injury kills a substantial amount of myocardial tissue, and therefore likely represents a clinically relevant therapeutic target.

Although MRI is emerging as a leading technique to measure the size of irreversible tissue damage, questions remain as to the best timing of imaging with respect to reperfusion. Mostly, it has been demonstrated that the area of hyper-enhancement after gadolinium injection (which delineates the area of infarction) can shrink by nearly 30% between day 5 and month 5 after AMI. This is likely due both to a diminution of oedema within the reperfused territory and to the remodelling of the infarcted myocardium.

2.4 Controlling the determinants of infarct size

The assessment of co-factors that determine infarct size is a major point. As clearly established in animal models by Reimer et al., the final infarct size following a prolonged ischaemia–reperfusion is predicted by: (i) the duration of ischaemia, (ii) the size of the area at risk, and (iii) the amount of collateral circulation to the risk region during the ischaemic period. It is of paramount importance to measure all three factors in clinical infarct size reduction studies.

Although not always reliable, the duration of ischaemia is usually estimated by the time elapsed between the onset of chest pain and the opening of the culprit coronary artery by coronary angioplasty. It is widely accepted (mainly based on early thrombolysis, rather than PCI studies) that the amount of tissue salvage decreases and the prognosis worsens as the duration of ischaemia increases. Guidelines recommend that emergency reperfusion in STEMI patients should be performed when ischaemia time is <12 h. Of note, the impact of the duration of ischaemia on the amount of lethal reperfusion injury is poorly understood. Although it is intuitively accepted that the amount of irreversible myocardial damage due to reperfusion injury is proportional to the severity of ischaemia, the constant persistence of this relationship over the 12 h therapeutic range remains to be determined. In fact, Maninveldt et al. found in the rat model that ischaemic postconditioning was effective when ischaemia lasted >45 min, but was detrimental (i.e. increased infarct size) when coronary occlusion was of short duration. As for clinical practice, one may question whether PCI postconditioning or CsA is truly of interest when reperfusion occurs in <2 h, where infarct size would anyway be limited. On the other hand, these interventions might be of major interest when ischaemia lasts >6 h, where lethal reperfusion injury may well be more deleterious than expected, and where any additional myocardial salvage may be of important prognostic value. Further assessment of the amount of salvage according to time to revascularization in larger cohorts of postconditioned or CsA-treated patients is required to address this issue.

Evaluation of the amount of collateral flow to the ischaemic region is unfortunately very difficult in the human
myocardium, especially in emergency situations. In theory, the best technique to measure myocardial blood is PET scan, which is impossible to apply in emergency settings. Other techniques, including contrast echocardiography, SPECT, or MRI are feasible yet difficult to apply in such clinical settings, because of limited access to nuclear medicine facilities, and mostly because they might delay the PCI procedure. To attenuate this limitation, we decided in our PCI postconditioning and CsA proof-of-concept trials to exclude patients with overt collateral circulation at the admission coronary artery angiography: overall, this subpopulation with native collaterals to the area at risk averaged 15.2% of all included patients. It is well demonstrated that these patients develop small infarcts and would likely get little, if any, benefit from a further intervention. In fact, analysis of our database in which these patients with collateral circulation were entered and had CK release measurements revealed that they displayed a 55% reduction of infarct size when compared with controls, i.e. much more than either postconditioning or CsA treatment would have afforded. This is in agreement with infarct size studies performed in the dog model which has collateral circulation. The plots of infarct size vs. regional myocardial blood flow clearly show that hearts with more than ~15% of the myocardial blood flow in the remote non-ischaemic territory during a prolonged ischaemia develop small infarcts; infarct size in these hearts with collateral circulation is half that seen in postconditioned hearts without collateral circulation.17

The determination of the size of the area at risk is a major issue. In animal models that have little collateral circulation (e.g. pig, rabbit, rat, mouse), area at risk variations can predict 70–90% of the final infarct size.67 In AMI patients, the more appropriate technique is probably sestamibi-SPECT.56 It allows a delayed (<6 h) imaging with respect to the time of injection, which in this case could be performed before PCI revascularization. It however requires that nuclear medicine imaging could be performed on a 24 h basis, which unfortunately is not possible in most PCI centres. We used LV angiography to measure the area of abnormal contraction, a surrogate for the area at risk, as described by Feidt et al.69 We found that there was a good correlation between infarct size (as measured by CK release) and the area of abnormal contraction, very similar to the relationship reported in animal models (Figure 3). New imaging modalities of MRI, including T2-weighted sequences, have been reported as able to assess the area at risk in experimental preparations.70 Delineation of the area at risk by T2-weighted MRI is based on the increased amount of water in the myocardium at risk due to tissue oedema after reperfusion. Although this technique may be useful in several situations, it has a major limitation when applied to infarct size reduction studies. In fact, most infarct size reduction treatments, and particularly ischaemic postconditioning, have been shown to reduce oedema in the reperfused myocardium.17 Therefore, any imaging technique measuring oedema would underestimate the area at risk in the treated group (but not in the control group) and could artificially increase the infarct size/risk region ratio; this might lead to missing a protective effect. Assessment of the size of the area at risk brings us important information. First, it enhances the sensitivity for the group comparison so that sample size can be significantly reduced.

Secondly, it demonstrates that the patients who benefit the most from the therapeutic intervention are those who display the larger area at risk (Figure 3). In the mean time,
it shows that patients with small area at risk get little benefit from the therapeutic intervention aimed at attenuating lethal reperfusion injury, be it postconditioning or CsA. This information is of crucial importance when designing large-scale trials aimed at investigating the ability of any therapeutic interventions supposed to limit infarct size.

2.5 Controlling the conditions of reperfusion

The assessment of the efficacy of any intervention aimed at reducing lethal reperfusion injury requires to control the conditions of reperfusion as strictly as possible. Postconditioning and CsA have been shown to attenuate lethal reperfusion injury in conditions where: (i) the coronary artery was fully occluded before reperfusion, (ii) treatment was applied no later than the first minute of reperfusion. Most recent large-scale morbidity–mortality clinical trials performed in STEMI patients revascularized by PCI included patients with TIMI flow grade at admission ranging from 0 to 3. In contrast, we included only patients with a fully occluded culprit coronary artery at admission (TIMI flow grade 0–1). TIMI 0–1 patients are those with the higher risk of developing a large infarct, i.e. more prone to benefit from postconditioning or CsA. Experimental studies have demonstrated that the therapeutic window for lethal reperfusion injury is restricted to the first minutes of reperfusion. STEMI patients with a TIMI flow grade >1 at the initial coronary angiography have indeed had a spontaneously reperfusion before admission; in this case, the therapeutic intervention will necessarily come after the therapeutic window for intervention has closed. However, whether this therapeutic window is as narrow in the human heart as in studied animal species is still unknown. As suggested by recent preliminary experimental studies, it is possible that even delayed interventions may provide some infarct size reduction.

We used the direct stenting technique in our three studies. It has been clearly demonstrated the low-flow, low-pressure, or staged reperfusion can postcondition the heart. Facing a fully occluded coronary artery, after letting the guide wire cross the lesion, most PCI cardiologists would perform a pre-dilatation in order to envision the aspect of the stenosis as well as the distal coronary artery bed. Some might repeat balloon inflations before finally stenting the culprit lesion: this repetition of balloon inflations and deflations may well trigger postconditioning. Doing so in the control group would have limited our ability to demonstrate the potential benefit of postconditioning or CsA. In contrast, direct stenting immediately after passing the guide wire through the lesion, which often allows a minor reflow and visualization of the stenosis, more closely mimics the type of reperfusion used in experimental models.

The choice of the specific postconditioning algorithm with 5 min episodes of ischaemia–reperfusion was arbitrary, based on our experience in the rabbit heart. We took great care to perform the first balloon inflation <1 min after direct stenting, since preclinical evidence suggested that postconditioning protection was lost when the initial brief ischaemia was delayed after reflow.

3. Conclusion: from proof-of-concept to clinical application

Phase II clinical trials, especially when including a limited number of patients, are not sufficient for clinical application. One may consider that ischaemic postconditioning by PCI and CsA administered at the onset of reperfusion are safe and can reduce infarct size in STEMI patients. Limited data suggest that PCI postconditioning, by reducing infarct size, improves the recovery of contractile function at 1 year post-infarction. However, a widespread use of these cardioprotective therapies requires the demonstration that their application to a large number of patients improves clinical outcomes. Obviously, one might expect that infarct size reduction should improve survival and prevent the development of heart failure. In this regard, we are currently putting together a large-scale, multicentre, randomized trial to determine whether cyclosporine might improve clinical outcome in STEMI patients.

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