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New and revisited approaches to preserving the reperfused myocardium

Robert A. Kloner^{1,2}, David A. Brown^{3,4,5}, Marie Csete^{1,6}, Wangde Dai^{1,2}, James M. Downey⁷, Roberta A. Gottlieb⁸, Sharon L. Hale¹, and Jianru Shi^{1,2}

¹Cardiovascular Research Institute, Huntington Medical Research Institutes, 99 North El Molino Avenue, Pasadena, California 91101, USA

²Division of Cardiovascular Medicine and Department of Medicine, Keck School of Medicine, University of Southern California, 1975 Zonal Avenue, Los Angeles, California 90033, USA

³Department of Human Nutrition, Foods, and Exercise, 1981 Kraft Drive, Blacksburg, Virginia 24060, USA

⁴Virginia Tech Center for Drug Discovery, Virginia Tech, 1981 Kraft Drive, Blacksburg, Virginia 24060, USA

⁵Virginia Tech Metabolic Phenotyping Core, Virginia Tech, 1981 Kraft Drive, Blacksburg, Virginia 24060, USA

⁶Department of Anesthesiology, Keck School of Medicine, University of Southern California, Los Angeles, California 90017, USA

⁷Department of Physiology and Cell Biology, University of South Alabama, 5851 USA Drive North, Mobile, Alabama 36688, USA

⁸Department of Medicine, Barbra Streisand Women's Heart Center, Heart Institute of Cedars-Sinai, Cedars-Sinai Medical Center, 127 South San Vicente Boulevard, Los Angeles, California 90048, USA

Abstract

Early coronary artery reperfusion improves outcomes for patients with ST-segment elevation myocardial infarction (STEMI), but morbidity and mortality after STEMI remain unacceptably high. The primary deficits seen in these patients include inadequate pump function, owing to rapid infarction of muscle in the first few hours of treatment, and adverse remodelling of the heart in the months that follow. Given that attempts to further reduce myocardial infarct size beyond early

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Correspondence to: R.A.K. Cardiovascular Research Institute, Huntington Medical Research Institutes, 99 North El Molino Avenue, Pasadena, California 91101, USA. robert.kloner@hmri.org.

Author contributions

R.A.K., D.A.B., M.C., W.D., J.M.D., R.G., and J.S. researched data for the article. R.A.K., D.A.B., M.C., J.M.D., and R.G. wrote the article and substantially contributed to the discussion of its content. R.A.K., D.A.B., M.C., J.M.D., R.G., S.L.H., and J.S. reviewed/edited the manuscript before submission.

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reperfusion in clinical trials have so far been disappointing, effective therapies are still needed to protect the reperfused myocardium. In this Review, we discuss several approaches to preserving the reperfused heart, such as therapies that target the mechanisms involved in mitochondrial bioenergetics, pyroptosis, and autophagy, as well as treatments that harness the cardioprotective properties of inhaled anaesthetic agents. We also discuss potential therapies focused on correcting the no-reflow phenomenon and its effect on healing and adverse left ventricular remodelling.

Early reperfusion with percutaneous transluminal coronary intervention has been shown to be effective in reducing myocardial infarct size and improving cardiac function, but data from clinical trials to evaluate the efficacy of adjunctive therapies in further reducing myocardial infarct size have been disappointing^{1,2}. Consequently, despite the improvements in time-to-reperfusion for ST-segment elevation myocardial infarction (STEMI) and the use of evidence-based therapies during hospitalization in the past decade, in-hospital mortality for STEMI has not declined³. According to the AHA, 30-day mortality in patients presenting with myocardial infarction (MI) is as high as 7.8–11.4%⁴. Up to 18% of men and 23% of women aged <45 years will die within 1 year of their first MI event; these rates increase to 36% and 47%, respectively, up to 5 years after the initial event. In addition, among the survivors in this population, 16% of men and 22% of women will develop heart failure⁴. Several reasons have been proposed to explain why adjunctive therapies have not been effective in reducing infarct size and improving outcomes after MI in the clinical trial setting, including the lack of reproducibility and rigour in preclinical studies; use of preclinical models that do not adequately reflect clinical comorbidities such as advanced age; and administration of pharmacological agents too late to have an effect on infarct size. Furthermore, antiplatelet agents such as P2Y₁₂-receptor blockers are commonly prescribed to these patients before primary angioplasty, and might mask the protective postconditioning effects of adjunctive therapies⁵. In the setting of acute MI, these antiplatelet agents help to maintain vessel patency after percutaneous coronary intervention (PCI). In addition to their effects on platelet aggregation, these drugs are powerful postconditioning mimetics, and act on the same protective signalling pathways that are activated during ischaemic preconditioning or postconditioning^{6,7}. As a consequence, therapeutic strategies that target ischaemic preconditioning⁸ or postconditioning⁶ as a means for cardioprotection after MI did not reduce infarct size in animal hearts beyond the effects of the antiplatelet agents. The first clinical trial to evaluate the efficacy of ischaemic postconditioning after acute MI reported promising findings, but subsequent trials that were conducted after platelet inhibitors came into widespread use in patients undergoing PCI yielded either neutral results, or demonstrated only marginal benefit with postconditioning^{5,9,10}. Although the use of platelet inhibitors might not be the only confounding factor in these studies, any intervention that cannot provide additional cardioprotective effects beyond that of an antiplatelet agent in the setting of MI (as seen in the animal studies) is unlikely to improve outcomes. Remote ischaemic conditioning — whereby short, serial episodes of ischaemia and reperfusion applied to a (limb) vascular bed confer global protection against subsequent ischaemia–reperfusion injury — has been shown to reduce myocardial infarct size in several contemporary clinical trials and meta-analyses^{11–14}. The mechanisms involved in remote ischaemic conditioning are currently unknown and have not been tested against platelet inhibitors.

In light of these observations, an optimal strategy for preserving the myocardium after MI will be one that mitigates a factor contributing to cell death in the reperfused heart, but is not targeted by platelet inhibitors. For example, mild hypothermia and sodium–hydrogen exchange blockers offer additive protection against infarction when combined with the P2Y₁₂ inhibitor cangrelor in rats; the use of all three strategies in combination is three times as protective against infarction compared with the use of the platelet inhibitor alone⁸. Unfortunately, mild hypothermia and sodium–hydrogen exchange blockers seem to target ischaemic tissue rather reperfused tissue, and thus these strategies must be applied during ischaemia for optimal efficacy¹⁵. Although investigations exploring the effects of rapid cooling (either soon after admission or even in the ambulance to shorten the normothermic ischaemic time) are ongoing¹⁶, a better solution would be to develop an intervention that is effective when administered around the time of reperfusion. Future investigations of cardioprotective agents should include experiments in animal models with common clinical comorbidities and in combination with platelet inhibitors to identify therapies most likely to translate to the clinical situation.

Infarct size reduction is not the only therapeutic goal after MI. Long-term therapy with angiotensin-converting-enzyme inhibitors, angiotensin-receptor blockers, and/or β -blockers reduces adverse left ventricular (LV) remodelling, but has only modest effects on mortality¹⁷. Whereas stem cell therapy has shown promise in some studies, most of the larger clinical trials yielded either neutral findings or demonstrated very limited improvements in LV ejection fraction after MI¹⁸. The promising preclinical results in periprocedural MI stem cell therapy have not yet translated into clinical benefit for patients¹⁸. However, cell mimetic therapies such as nanoparticles¹⁹ and synthetic stem cells²⁰ engineered to mimic stem cell trophic effects are in preclinical stages of development. This field of research highlights the major unmet need for noncellular therapies that can improve cardiac structure and function after MI, perhaps in association with cell therapy. In this Review, we consider several promising approaches for the preservation of cardiac structure and function after MI that are not currently part of the clinical armamentarium (FIG. 1). This Review takes a broader approach than previously published Reviews on cardioprotection focused on preconditioning, postconditioning, and remote ischaemic conditioning, plus their pharmacological mimetics^{1,2,21,22}. We discuss cardioprotective mechanisms that target mitochondrial bioenergetics, anaesthetic preconditioning, pyroptosis, autophagy, and the no-reflow phenomenon. All these therapies involve mitochondrial function to some extent, such that progress in each of the approaches should inform the others. Indeed, a newly listed clinical trial involving the transplantation of autologous skeletal muscle mitochondria epicardially in children undergoing extracorporeal membrane oxygenation after ischaemic injury points to the emerging importance of bioenergetic approaches for heart disease²³.

Targeting the mitochondria

Bioenergetics and ROS generation

The pathophysiology of cardiac reperfusion injury is complex, and varies over time after injury. Altered ion homeostasis, inflammation, activation of adaptive signalling pathways,

metabolic substrate utilization shifts, and mitochondrial dysfunction are among the many factors that contribute to electromechanical dysfunction and tissue injury. The mitochondria are complex structures that undergo biogenesis, fission, and fusion independent of cell cycling. As central hubs of cellular energetics and redox signalling, the mitochondria influence virtually all cellular functions, particularly cell survival. They have been identified as a drug target in ischaemic heart disease, given the negative effects of increased generation of reactive oxygen species (ROS), abnormal cellular energetics, and impaired mitochondrial ion homeostasis on cardiac structure and function (FIG. 2; BOX 1)^{24–27}. Mitochondrial functional aberrations are often inter-related, such as imbalances in calcium and sodium ion levels, opening of energy-dissipating channels or pores, and increased production of ROS^{27–30}.

Box 1

Ischaemia–reperfusion injury: targeting the mitochondria

- TEMPO conjugates: XJB-5-131, mito-TEMPO
- Permeability transition pore blockers: cyclosporine A, TRO-40303, platelet inhibitors
- Exogenous quinones: idebenone, EPI-743, mitoQ
- Transient/reversible electron transport chain inhibition: rotenone, mitoSNO, metformin
- Superoxide dismutase mimetics: EUK8, EUK134, MCI-186, M40403, Me2DO2A, Mn-TBA
- Attenuate release of mitochondrial DNA to lower pyroptosis
- Stimulate mitophagy/autophagy (?)
- Sustain mitochondrial membrane potential to support endogenous scavenging defences and ATP production

Formation of excess reactive oxygen intermediates, many of which originate from within the electron transport system and the mitochondrial matrix^{31,32}, NADPH oxidase³³, and catecholamine catabolism via monoamine oxidases^{34,35}, cause cellular pathology in many acute and chronic diseases. A small amount of ROS production is essential for normal physiological signalling, but at higher levels, ROS are toxic^{36–41}. During acute reperfusion injury, the rate of ROS production substantially outpaces the capacity of endogenous scavengers to reduce them. A number of strategies to eliminate ROS-mediated damage in the early reperfusion window have been clinically tested, but with limited success^{42–46}. A major source of mitochondrial ROS in early reperfusion seems to be from increased succinate levels during the ischaemic period⁴⁷, which is postulated to produce ROS by ‘reverse electron transfer’. ROS are released from mitochondria into respiratory complexes called supercomplexes, functional aggregates of the electron transport system that are essential for normal electron flux^{48–51}. Fewer respiratory supercomplexes are formed in the setting of acute⁵² and chronic^{53,54} cardiac pathologies. Since ROS production seems to be inversely

related to supercomplex formation⁵⁵, the loss of supercomplexes might be directly linked to augmented ROS production in a 'vicious cycle'. Interventions that promote sustained generation of supercomplexes might have the potential to reduce myocardial injury during ischaemia and reperfusion²⁵.

Direct scavengers of ROS—Direct scavenging of radicals has long been proposed as a potential treatment for reperfusion injury^{25,27}. Direct scavengers that lessen mitochondrial radical accumulation, such as the scavenger 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO; targeted to mitochondria via gramicidin XJB-5-131 (REFS 52,56)) or triphenylphosphonium (Mito-TEMPO)⁵⁷ are cardioprotective in preclinical models. The hydroxyl scavenger 3-methyl-1-phenyl-2-pyrazolin-5-one (MCI-186)⁵⁸ and several newer superoxide dismutase mimetics such as EUK8/EUK134 (REFS 59–61), M40403 (REF. 62), Me2DO2A⁶³, and Mn-TBPA⁶⁴ that convert (via reduction reactions) harmful superoxide molecules into oxygen or hydrogen peroxide are cardioprotective in animal models of ischaemia–reperfusion. Unfortunately, these promising preclinical results have not been replicated in clinical trials^{65,66}. Coenzyme Q₁₀ (also known as ubiquinone) is an endogenous, free-radical-scavenging antioxidant reported to be beneficial in preclinical models of ischaemia–reperfusion^{67,68}. Coenzyme Q₁₀ administration in patients with heart failure significantly reduced cardiac mortality⁶⁹. Quinones that target mitochondria, such as mitoQ⁷⁰ and SkQ⁷¹, are showing promise in preclinical studies. However, at higher doses, cation conjugates can inhibit mitochondrial function⁷². Ideally, future exogenous scavengers should be engineered for improved tissue permeability and more specific intracellular or mitochondrial targets.

Interventions that inhibit mitochondrial respiration—Transient, partial inhibition of mitochondrial respiration during the vulnerable early-reperfusion phase might also be cardioprotective by limiting mitochondrial ROS production. Rotenone⁷³ and several different biguanides (such as metformin)⁷⁴ inhibit complex I of the mitochondrial respiratory chain, and have been shown to be cardioprotective when administered after ischaemia^{75,76}. Notably, however, rotenone is a well-known neurotoxin, and its systemic effects are likely to limit its use in this setting. Likewise, inhibition of complex II (succinate dehydrogenase) with malonate (or the cell-permeable precursor dimethyl malonate) has been shown to reduce ROS and decrease ischaemia–reperfusion injury^{47,77,78}. Furthermore, post-translational modification of electron transport complexes by S-nitrosothiols (mito-SNO) that are engineered to selectively block ROS protects mitochondrial function and lessens reperfusion injury in rodents^{79–83}. The safety of salvaging tissue by transiently blunting mitochondrial bioenergetics must be determined before these approaches can be advanced to clinical trials. Furthermore, methods to target these strategies specifically to the heart (to avoid inhibiting respiration in other organ systems) must also be identified.

Interventions targeting post-translational modifications—Post-translational modifications of electron transport complexes and endogenous ROS-scavengers via sirtuins⁸⁴ and O-GlcNAc modifications⁸⁵, or succinylation⁸⁶ might help in the search for novel pharmacological interventions to treat post-ischaemic injury. Given that the adaptor protein p66^{Shc} catalyses ROS formation via cytochrome *c*, several studies have investigated the potential role of p66^{Shc} as a regulator of myocardial injury in murine models of cardiac

ischaemia and reperfusion. Genetic loss-of-function studies have yielded opposing findings regarding cardiac injury^{87,88}. Isolated hearts from p66^{Shc} knockout mice were protected against acute reperfusion injury⁸⁸. However, a subsequent investigation assessing ischaemia–reperfusion *in vivo* found that neither knockout mice nor transient transfection with p66^{Shc} small interfering RNA imparted cardioprotection⁸⁷, and was actually deleterious. These differences might be related to inherent discrepancies between *in vivo* versus *in vitro* studies, as well as differing durations of ischaemic insult across studies. Further research is warranted to fully understand the role of p66^{Shc} signalling and post-translational modifications in reperfusion injury⁸⁹.

Interventions targeting mitochondrial membrane potential—Interventions to improve mitochondrial function often focus on mitochondrial membrane potential (ψ_m). The transmembrane potential of mitochondria is established by electron-dependent proton pumping, and serves as the major energetic driver for the replenishment of ATP, cellular redox buffering, and ion/metabolite transport. Loss of ψ_m during cardiac ischaemia–reperfusion is thought to be directly related to cellular injury and electromechanical dysfunction, as seen in *in vitro* models and intact hearts subjected to ischaemia–reperfusion injury^{28,43,90–93}. Aon and colleagues hypothesized that pathophysiological ROS overflow occurs at both minimal and maximal ψ_m ⁹⁴. Highly polarized ψ_m is observed in mitochondria that are ‘idling’ (that is, mitochondrial respiration with little to no ATP turnover), suggesting that slight uncoupling might protect mitochondria by reducing ROS formation⁹⁵. Although this strategy has been used to treat other diseases influenced by mitochondrial dysfunction⁹⁶, mild uncoupling in the heart did not markedly reduce mitochondrial ROS production⁹⁷, and actually led to increased ROS production in isolated myocytes⁹⁸. Numerous studies have shown that cardioprotection is associated with sustained, polarized ψ_m ; this energized ψ_m is essential during metabolic insult to replenish endogenous antioxidants via NADPH-linked reactions, which allows for the downstream detoxification of free radicals^{99,100}. Cardioprotection inferred by sustained ψ_m has been observed after exercise preconditioning⁹¹, after administering serofendic acid (a cardioprotective substance found in fetal calf serum which inhibits the oxidant-induced mitochondrial death pathway¹⁰¹), or by blocking energy-dissipating ion channels within the mitochondrial inner membrane^{102,103}.

A reduction in ψ_m is an early step in programmed cell death¹⁰⁴, a process considered irreversible after the apoptotic cascade is initiated. However, even after activation of caspase 3 and DNA nicking, some cells (including primary cardiomyocytes) can recover through a process known as anastasis¹⁰⁵. Caspase 3 is used extensively in normal development and in adult organ remodelling, so strategies that can activate the nonapoptotic properties of caspase 3 after cellular injury might provide a potential intervention for cardioprotection after ischaemia–reperfusion¹⁰⁶.

The mitochondrial PTP

Potential strategies to preserve mitochondrial bioenergetics by targeting the permeability transition pore (PTP) have received considerable interest, and have been the subject of numerous reviews^{107–110}. Inhibition of PTP at reperfusion is thought to be a primary

mechanism by which ischaemic preconditioning and postconditioning protect the heart^{108,111}. As calcium and ROS are postulated to increase the probability of the opening of the PTP, strategies that reduce ROS and lower mitochondrial calcium overload have been extensively explored as cardioprotective interventions^{25,27}. Paradoxically, transient PTP opening seems to reduce mitochondrial calcium overload, analogous to a 'pressure release valve', whereby mitochondrial calcium decreases upon transient PTP opening¹¹². This mechanism works to reset mitochondrial calcium concentration, and is postulated to prevent the sustained, large conductance, open conformation of PTP that is associated with pathological injury and cell death. Studies published in the past 6 years suggest that the PTP is also comprised of ATP-synthase (complex V) dimers and associated regulatory proteins^{113,114}.

Use of the PTP blocker cyclosporine A for the treatment of acute coronary syndromes initially showed promise in small trials^{115,116}, but a larger multicentre trial yielded neutral findings¹¹⁷. TRO-40303, a ligand of the putative PTP component translocator protein (also called the peripheral benzodiazepine receptor), did not impart any cardioprotective benefits in patients with MI¹¹⁸. The underlying factors involved in these neutral findings are clearly multifaceted; of note, however, most patients involved in PCI trials since 2005 have received platelet inhibitors as a standard-of-care therapy. As platelet inhibitors can independently protect the heart by inhibiting the PTP^{6,7,119}, patients enrolled in trials that assessed the efficacy of PTP blockers might already be 'conditioned' against injury. Importantly, cyclosporine and this 'conditioning' effect do not prevent PTP opening, but instead simply raise the threshold for opening. The development of strategies targeting hexokinase (a regulator of PTP opening) to mitochondria^{120–122}, as well as more specific PTP blockers that target mitochondria¹²³ might lead to new therapies for the treatment of ischaemia–reperfusion injury. Several other mitochondrial approaches to protect the heart in this setting are also under development, including methods to deliver autologously derived mitochondria^{124–126}, new mitochondria-targeting peptides^{43,127–130}, strategies that target calcium-activated mitochondrial potassium channels¹³¹ or block ATP-sensitive potassium channels^{132–134}, and approaches that block the inflammation caused by mitochondrial DNA after MI^{135,136}.

Challenges and opportunities

Given the role of the mitochondria in reperfusion injury, novel compounds targeted towards altering mitochondrial bioenergetics might reduce myocardial injury. However, delivery of these compounds to mitochondria poses as a daunting problem. Timing is a major concern, because the compound must reach therapeutic levels during the appropriate window, which is often early in reperfusion. Given the importance of minimizing 'door-to-balloon' times, achieving therapeutic levels in time with systemic delivery is challenging; direct coronary catheter administration might be more feasible for reducing acute injury. Bioavailability of the compound poses a further challenge, because the compound must resist degradation (or sequestration) in the circulation, traverse the capillary network and cross cell membranes, and then localize to mitochondria. Medicinal chemistry considerations for mitochondria-targeted therapies include whether the targeting moiety is cleaved inside cells, as well as appropriate chemical linkers and positions to which the cargo should be attached on the

parent mitochondria-targeting molecule. Despite these obstacles, a number of putative mitochondria-targeting strategies are emerging.

The highly negative ψ_m of healthy mitochondria is frequently utilized as an attractant to deliver mitochondrial cargo via positively charged carriers. The most well-characterized conjugate family to date uses triphenylphosphonium, a cationic lipophile, to deliver therapies to mitochondria^{30,137–139}. A similar approach is being developed using plant-derived cell-permeable cations such as berberine or palmatine^{140,141}, although some of these compounds can inhibit respiration at low micromolar concentrations⁷².

Peptide conjugates are another emerging therapy targeted to mitochondria. Multiple peptide sequences containing alternating cationic and hydrophobic amino acids show cell permeability and mitochondrial localization, and are being developed as delivery vectors¹⁴². Examples of these peptides include mitochondria-penetrating peptides^{143,144} and several of the Szeto-Schiller peptides¹⁴⁵. The ROS scavenger XJB-5-131 conjugates the scavenging TEMPO moiety to mitochondria via a gramicidin peptide sequence⁵⁶; this sequence might be useful to deliver other cargo to mitochondria. Nanoparticles have also been developed to reduce cardiac mitochondrial injury^{146,147}, although the nanoparticle vector might be more important for enhancing cellular uptake in damaged cells than for mitochondrial localization *per se*. However, cardiac toxicity has been observed with several types of nanoparticles^{148,149}, indicating that more research is needed to develop these agents. Continued investigation into the safety and efficacy of vectors that deliver cargo to mitochondria is an exciting area for cardioprotective therapies.

Anaesthetic preconditioning

Clinical studies of anaesthetic conditioning of the heart have focused almost entirely on preconditioning and on patients undergoing cardiac surgery, for practical reasons — anaesthesiologists are present at the critical time before the coronaries are reperfused. Substantially less work on anaesthetic preconditioning has been conducted in MI models, and on anaesthetic postconditioning after cardiac surgery, despite the technique having therapeutic potential in these settings as well. Similar to ischaemic preconditioning, short exposure of the heart to halogenated volatile anaesthetics offers cardioprotection against subsequent ischaemic or ischaemia–reperfusion injury¹⁵⁰. Pharmacological studies suggest that the mechanisms of anaesthetic and ischaemic preconditioning are similar. Volatile anaesthetic preconditioning is complex, and involves agents that increase mitochondrial ROS production; activate protein kinase C (PKC), extracellular signal-related kinase (ERK1/2, also known as mitogen activated protein kinase 3 and 1), and Akt (also known as Rac- α serine/threonine-protein kinase); open K_{ATP} channels acutely; and reduce myocardial oxygen consumption^{151–153}. Given these signalling pathways are the same as those targeted by P2Y₁₂ platelet inhibitors, anaesthetic preconditioning is not likely to provide additional protection in patients undergoing PCI who are treated with platelet inhibitors. Patients who are naive to these drugs, such as those undergoing cardiac surgery, are more likely to respond to anaesthetic preconditioning.

Data from preclinical studies suggest that activation of aldehyde dehydrogenase 2 by mitochondrial protein kinase C- ϵ (PKC ϵ) is necessary for effective anaesthetic preconditioning¹⁵⁴, as well as inactivation of cardiac L-type calcium channels¹⁵⁵ and increased production of heat shock protein 90 (and its induction of endothelial nitric oxide synthase)¹⁵⁶. Longer-term cardiac protection by anaesthetic preconditioning might be mediated through downstream effectors of hypoxia-inducible factor 1 (REF. 157), but its mechanism of action in this setting is unclear. The first double-blind, placebo-controlled trial of anaesthetic preconditioning in patients undergoing cardiac surgery used sevoflurane (5 min inhalation before cardioplegia) on a background of opioid maintenance anaesthesia¹⁵⁸. Standard clinical laboratory evidence of MI (cardiac enzymes) were unaffected by this preconditioning regimen, but preconditioning reduced B-type natriuretic peptide levels. Atrial biopsies showed translocation of PKC ϵ to the sarcolemma, mitochondria, and intercalated disks of cardiomyocytes after preconditioning¹⁵⁸. Unsurprisingly to anaesthesiologists, treated patients required substantially more pressor support during preconditioning, but still had better glomerular filtration rates after CABG surgery than untreated patients, suggesting a preconditioning effect on the kidneys. Subsequent studies also reported cardioprotection with use of various inhaled anaesthetics after CABG surgery, but these effects were not potent enough to change major outcome variables^{159,160}. In theory, an increase in dosage should lead to an increase in potency, but acute increases in the concentration of inhaled anaesthetics can lead to hypotension and cardiac depression. Similarly, intraoperative remote ischaemic preconditioning (via cuff inflation–deflation on the leg) added to isoflurane preconditioning did not increase the potency of anaesthetic preconditioning, and might even blunt the anaesthetic effect¹⁵⁹.

Challenges and potential confounders

With a gradual increase in experience with application of anaesthetic preconditioning during CABG surgery, researchers found that the benefits of anaesthetic preconditioning for cardiac ischaemic events in animal models were not readily translated to humans. The heterogeneity of patients is often unmasked when translating studies from inbred rodent models to the clinic. Furthermore, most animal studies are performed in young animals, whereas most patients undergoing CABG surgery are not young, and senescent hearts are difficult to precondition, even in animal models¹⁶¹. Variables that can affect outcomes after cardiac surgery, such as other drugs, fluids, electrolytes, transfusions, and physiological gases administered, can be very difficult to control for in clinical studies. Furthermore, the inflammatory and oxidative stress-induced damage caused by ischaemia–reperfusion injuries are difficult to avoid in the operating room, as are presurgical comorbidities.

Hyperglycaemia, in particular, blunts anaesthetic preconditioning, in part by disruption of mitochondrial bioenergetics¹⁶². A study using human induced pluripotent stem cell (iPSC)-derived cardiomyocytes showed that opening of mitochondrial potassium channels was disrupted in the presence of high glucose levels¹⁶³. Given the difficulty in designing clinical trials to assess the efficacy of anaesthetic preconditioning, and the slow progress in optimizing this technique owing to the complexity of cardiac surgery, human iPSC-derived cardiomyocytes are an important platform for probing anaesthetic preconditioning. These cells provide an opportunity for rigorous assessment of issues such as timing and amount of

preconditioning, changes in molecular mediators of preconditioning as a function of genetic background and the cellular microenvironment, and potential synergistic combinations of anaesthetics and other drugs, all of which are nearly impossible to measure in the clinical setting. Combined with standard preclinical models, human iPSC technology should provide a platform for revisiting clinical anaesthetic preconditioning that allows assessment of the mechanisms underlying the anaesthetic effects.

Pyroptosis and the innate immune system

A study published in 2015 reported that strategies to prevent the escape of mitochondrial DNA (mtDNA) from ruptured mitochondria can be cardioprotective in the setting of infarction when applied just before reperfusion¹³⁶. Two approaches were tested: the first involved mitochondrial-directed glycosylase, which removes oxidized bases from mtDNA created during reperfusion, thus favouring mtDNA repair over its release; and the second strategy involved use of intravenous DNase I, which degrades any extracellular DNA. The effect of both of these interventions on infarct-related injury were similar to that of conditioning, and most importantly, offers additive protection when combined with a P2Y₁₂ inhibitor in animal models, suggesting that mechanism of mtDNA toxicity cannot be addressed by P2Y₁₂ inhibitor administration alone. Therefore, strategies targeting released mtDNA are likely to be cardioprotective in patients who are already undergoing P2Y₁₂ inhibitor treatment, and warrant further investigation.

The most likely explanation for the toxicity of mtDNA is inflammation, one of the earliest proposed mechanisms of cell death from ischaemia–reperfusion injury. In the 1980s, studies of inflammatory responses concentrated on leukocytes, because they quickly accumulate in large numbers in ischaemic tissue¹⁶⁴. Although leukocytes are involved in cell death after MI, most approaches to prevent their accumulation did not limit infarction, and interest in this strategy subsequently waned¹⁶⁴. In 2014, a reassessment of inflammation in ischaemia focused specifically on the innate immune system¹⁶⁵. FIGURE 3 shows a diagram of the NLRP3 (also known as NACHT, LRR, and PYD domains-containing protein 3) inflammasome, an important component of the innate immune system. Invading pathogens release pathogen-associated molecular patterns (PAMPs) that signal the presence of sepsis. These PAMPs initiate interleukin and interferon production through the Toll-like receptor (TLR) system, whereby TLR4 recognizes lipopolysaccharide and TLR9 recognizes bacterial DNA. This sequence of events is often referred to as signal 1. PAMPs also trigger the assembly of the inflammasome, in a process known as signal 2. NLRP3 combines with the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and pro-caspase 1 to form active caspase 1. Caspase 1, in turn, cleaves pro-interleukins to form active IL-1 β and IL-18 (REF. 166). Caspase 1 can also liberate the N-terminal segment of gasdermin D, which creates pores in the cell membrane, in a process known as pyroptosis. These membrane pores allow cytokines to exit the cell and call inflammatory cells to attack the invader, but they also inevitably kill the cell^{167,168}.

Sepsis is not the only trigger for inflammasome formation. Damage-associated molecular patterns (DAMPs) from injured tissue, such as mtDNA, can also trigger the formation of inflammasomes¹⁶⁹. Reperfusion causes a burst of oxygen radicals in the mitochondria,

which oxidizes bases in the mtDNA and promotes the opening of the PTP through which oxidized mtDNA fragments flow into the cytosol¹⁷⁰. With its many unmethylated stretches of DNA in which the frequency of a cytosine immediately followed by a guanine is high, these mtDNA fragments resemble bacterial DNA, are TLR9 ligands, and are potent inducers of the NLRP3 inflammasome¹⁷¹. Extracellular mtDNA is a potent proinflammatory DAMP in traumatic shock¹⁷² and in ventilator-induced lung injury¹⁷³.

Infusing mtDNA into the coronary artery increases infarct size in Krebs buffer-perfused rat hearts¹³⁶; similarly, mtDNA added to culture medium induces cell death in cultured cardiomyocytes¹⁷⁴, indicating that mtDNA triggers cytotoxic pathways in the heart's parenchymal cells rather than just in the circulating white cells. Investigations into the role of the NLRP3 inflammasome in ischaemic injury have yielded mixed findings, with some studies reporting increased resistance to infarction in hearts in which NLRP3 inflammasome has been genetically deleted^{175,176}, and others reporting neutral findings^{177,178}. Importantly, the NLRP3 inflammasome is not the only activator of caspase 1; at least three other similar inflammasome systems that activate caspase 1 have been described¹⁷⁹, which might explain why chronic deletion of the NLRP3 inflammasome might be compensated for by activation of another inflammasome. Several pharmacological NLRP3 inhibitors have been developed, which have all been effective in limiting infarct size in animal models^{180–182}.

Attempts to mitigate caspase 1-mediated damage have yielded more consistently positive findings. In 2001, a study involving human atrial trabeculae subjected to simulated ischaemia–reperfusion showed that inhibition of caspase 1 with a highly selective inhibitor (Ac-YVAD-cmk) preserved contractile function¹⁷⁶. IL-18 binding protein and an IL-1 receptor blocker were similarly protective, suggesting that interleukins were involved in mediating the injury. Ac-YVAD-cmk was also found to reduce infarct size in an open-chest rabbit model of surgically induced MI¹⁸³. Furthermore, caspase 1 overexpression in mice increased infarct size after ischaemia–reperfusion by 50%, whereas complete knock-out of caspase 1 reduced infarct size^{175,184}. The pan-caspase inhibitor z-VAD has also been reported to reduce infarct size^{185,186}; caspase inhibition using z-VAD was assumed to target apoptosis, a process which involves caspase 3 and caspase 9, but the action of caspase 1 would have also been inhibited. Unlike necrosis and pyroptosis, apoptosis does not result in rapid membrane failure and could not have accounted for the washout of dehydrogenase enzymes in the studies of myocardial ischaemia–reperfusion¹⁸⁷. However, the apoptotic process seems to be involved in the release of the mtDNA that triggers inflammasome activation¹⁸⁸. Caspase 1 not only produces cytotoxic cytokines, but also proteolytically cleaves several important glycolytic enzymes, including glyceraldehyde phosphate dehydrogenase¹⁸⁹. Loss of these glycolytic enzymes could contribute to infarction by impairing metabolic recovery during reperfusion. None of the aforementioned caspase 1 studies addressed the problem of possible redundancy with platelet inhibitors, but a study published in 2017 revealed that protection conferred by the highly selective caspase 1 inhibitor VX-765 is additive to the protective effects of the P2Y¹² blocker cangrelor¹⁹⁰.

Caspase 1 also inhibits autophagy (lysosomal-mediated degradation) of mitochondria (also known as mitophagy)¹⁹¹. Given that mtDNA can promote inflammation in response to ischaemia–reperfusion, mitophagy might be cardioprotective in this setting. Under normal

conditions, the body is careful not to release free mtDNA into the cytosol. When senescent mitochondria are eliminated by autophagy, they are enveloped in a vacuole and digested by enzymes such as DNase II. Complete knock-out of DNase II produces a heart failure phenotype, which could be rescued using a TLR9 blocker, indicating that mtDNA that escaped from the vacuole was responsible for the phenotype¹⁹². Stimulating mitophagy at a time when mitochondria will be injured by ischaemia–reperfusion might attenuate mtDNA release, and could be yet another explanation as to why autophagy can reduce ischaemic injury.

Autophagy as a new therapeutic target

Role in ischaemic preconditioning

Since the discovery of preconditioning in 1986, investigators have sought to identify pharmacological triggers, elucidate the contributing signal transduction pathways, and define the mechanisms involved¹⁹³. Autophagy is induced by many of the classic cardioprotective agents, including ischaemic preconditioning itself¹⁹⁴, as well as diazoxide¹⁹⁴, the adenosine A₁ receptor agonist 2-chloro-N(6)-cyclopentyladenosine¹⁹⁵, sulfaphenazole¹⁹⁴, rapamycin¹⁹⁶, valsartan¹⁹⁷, resveratrol¹⁹⁸, non-sirtuin histone deacetylase inhibitors¹⁹⁹, and remote ischaemic conditioning²⁰⁰. Many investigators have demonstrated that autophagy is cardioprotective^{201–203}, and several studies have demonstrated an absolute requirement for functional autophagy in cardioprotection^{194–197,204}. Furthermore, selective mitophagy has been shown to be essential for cardioprotection by ischaemic preconditioning²⁰⁵ and statins²⁰⁶. Despite some early contradictory studies^{207,208}, autophagy is now generally regarded as cardioprotective when initiated before ischaemic stress. Although clearance of damaged mitochondria is undoubtedly an important protective consequence of autophagy, autophagy is likely to be cardioprotective through other mechanisms as well, including sequestration and degradation of glycogen, and removal of protein aggregates^{209,210}.

Role in postconditioning

The role of autophagy in postischaemic conditioning is more controversial, in part because of the complex roles and interpretation of autophagy markers. Detection of an increase in autophagosomes by immunostaining or the widely used autophagosome marker LC3-II might indicate upregulation of autophagy, but could also mean impaired autophagosome clearance by lysosomes (impaired flux). Moreover, interventions that restore flux could be misinterpreted as downregulation of autophagy²¹¹. Therefore, interpretation of autophagy studies must take into account whether flux was assessed. Most, but not all, reports support a beneficial role for autophagy in postconditioning^{212–215}.

Harmful effects

Autophagy often has a protective role in the heart, but in some settings might also be detrimental. For instance, autophagic cell death, or autosis, has been reported in the setting of cerebral ischaemia, and abrogation of autophagy through gene silencing or chemical inhibitors can reduce cell death^{216,217}. Interestingly, a chemical screen identified cardiac glycosides (digoxin, digitoxigenin, and strophanthidin) as antagonists of autosis²¹⁸. In the heart, less is known about the settings in which autosis might occur. Beclin 1 has been

shown to be important in mediating autosis in the heart²¹⁹, and haploinsufficiency of Beclin 1 might be cardioprotective²²⁰.

Regulatory pathways

Autophagy is primarily regulated by the nutrient-sensing and energy-sensing pathways, namely mechanistic target of rapamycin (mTOR) and AMP-dependent protein kinase (AMPK), respectively. However, multiple other signal transduction pathways are also involved in autophagy, including forkhead box protein O1, p38 mitogen-activated protein kinase, and Akt 2 (also known as RAC- β serine/threonine protein kinase)²²¹. Importantly, autophagy and mitophagy are regulated by sirtuins, a family of NAD-dependent protein deacetylases^{222–224}. Numerous studies have revealed a role for microRNAs (miRNAs) in the regulation of cardiac autophagy; some of these miRNAs are in the circulation or are delivered via exosomes^{225–240}. The term ‘autophagoMIR’ has been used to designate miRNAs that regulate autophagy, and includes both suppressors and activators of autophagy (TABLE 1). Importantly, miR-144 delivered via exosomes has been implicated as a circulating effector of remote preconditioning²⁴¹. miR-30 targets Beclin 1, which has a dual role in initiation of autophagy, but also can interfere with autophagic flux²⁴². Therefore, downregulation of Beclin 1 can have unpredictable effects on autophagy, and autophagic flux should always be measured to allow for meaningful interpretation of autophagy studies.

Cardiac remodelling

The role of autophagy in cardiac remodelling is controversial, with numerous studies reporting evidence for a protective role^{209,243,244}, an adverse role²²⁰, or both^{199,245}. Autophagy has also been implicated in the pathological role of angiotensin II on hypertrophy²⁴⁶, but its role in this setting is somewhat controversial²⁴⁷, and might be related to a functional dichotomy of autophagy or the nonspecific criteria for defining upregulated autophagy. In the absence of measures of autophagic flux, misinterpretation of LC3 levels or autophagosome abundance is likely²¹¹. However, the roles for mitochondrial autophagy in cardiac homeostasis²⁴⁸, adaptation to stress^{204,249}, and handling of misfolded proteins²⁰⁹ are well established.

Lifestyle factors

Autophagy is a homeostatic process that evolved from the need to survive episodes of nutritional deprivation, and is invoked during sleep, when caloric intake is absent; chronic nutritional excess, however, eventually suppresses autophagy. Caloric restriction potently upregulates autophagy, and intermittent fasting — even in the absence of reduced caloric intake — can upregulate autophagy and benefit the heart^{250,251}. Similarly, time-restricted feeding (8 h per day) results in a period of fasting and can protect mice against the ill effects of a high-fat diet²⁵². A high-fat diet has been shown to impair autophagy^{253,254} and reduce ischaemic tolerance²⁵⁵, underscoring the importance of intact autophagic flux in cardioprotection. Like caloric restriction and intermittent fasting, exercise upregulates autophagy and is associated with cardioprotection^{256,257}. Exercise-induced autophagy is important for clearing protein aggregates and ameliorates cardiac proteinopathy; the beneficial effects of exercise were enhanced in mice with an overexpression of autophagy-related protein 7 (REF. 209).

Cardioprotective effects of autophagy

Autophagy fulfils many roles in the heart. During ischaemia, autophagy provides amino acids and monosaccharides (from glycogenolysis) for ATP production, and is also essential for clearance of protein aggregates that accumulate in both heritable proteinopathies and in acquired heart failure²⁰⁹. During both ischaemia–reperfusion and pressure overload, autophagy serves to sequester and degrade damaged mitochondria that might otherwise trigger apoptosis or necroptosis. Moreover, autophagy is important in preventing the activation of the innate immune system (TLR9 and NLRP3), which can be triggered by mitochondrial cardiolipin, mtDNA, and oxidized mtDNA^{188,192,258}. Finally, mitophagy is important for mitochondrial quality control. Loss of oxidative phosphorylation capacity, whether through protein damage or mtDNA mutations, results in lower membrane potential, and mitophagy selectively eliminates the least functional organelles. Emerging evidence reveals a link between mitophagy and mitochondrial biogenesis^{259,260}, so that after eliminating damaged organelles, a stimulus expands the mitochondrial pool to restore ATP production capacity. One important benefit of mitophagy might be the activation of mitochondrial biogenesis, which would facilitate mitochondrial quality control. Indeed, exercise training, known to upregulate autophagy and protect against MI, has also been shown to improve myocardial energy metabolism and enhance mitochondrial biogenesis²⁶⁰. In addition to intermittent fasting and exercise, drugs that enhance mitophagy and biogenesis are also cardioprotective, and include statins²⁶¹ and NAD, which activates sirtuins²⁶²; this list is likely to expand to include preconditioning and postconditioning agents.

Fragmented mitochondria have been observed in numerous disease states in the heart, and targeting mitochondrial dynamics might be another area for therapeutic development²⁶³. Inhibition of mitochondrial fission has been shown to protect cardiac cells from reperfusion injury²⁶⁴. Treatment with a pharmacological inhibitor of Drp1-dependent fission, mitochondrial division inhibitor 1 (Mdivi1), protects the heart when administered either directly²⁶⁴ or as a nanoparticle conjugate¹⁴⁶. These strategies are postulated to prevent mitochondrial outer membrane permeabilization, which is triggered by Bax translocation during ischaemia–reperfusion, resulting in cytochrome *c* release and the onset of cell death cascades²⁶⁵. However, Drp1 (the target of Mdivi1), has been shown to mitigate mitochondrial dysfunction and heart failure in a pressure-overload model²⁴³.

No-reflow as a therapeutic target

In the setting of acute MI, no-reflow is defined as a lack of perfusion to portions of the myocardium, despite reopening of the occluded infarct-related epicardial coronary artery^{266–268}. Therapies targeting no-reflow have the potential to further preserve cardiac structure and function following MI. In a canine model of proximal coronary artery occlusion of 90 min, the fluorescent dye thioflavin S, which stains endothelial cells receiving flow, failed to penetrate much of the subendocardium when injected into the vasculature after the proximal coronary artery was reopened²⁶⁹. Perfusion defects were observed as early as 10–12 s after the reopening of the proximal coronary artery and seemed to increase in size as the duration of the reperfusion phase was extended to several hours²⁷⁰, suggesting that no-reflow is a true form of reperfusion injury. Other techniques confirm the

phenomenon of no-reflow, including failure of carbon black and radioactive microspheres to penetrate portions of previously ischaemic myocardium^{269,271}.

What causes no-reflow and what is its importance in the pathophysiology of disease- Our group has studied the ultrastructural features of the myocardium within the anatomical no-reflow zone, and found localized swelling or blistering of the endothelium of small blood vessels and capillaries²⁶⁹. Membrane-bound intraluminal blebs, protruding from the endothelial cells, obstructed the lumen of small blood vessels. Mitochondrial abnormalities might contribute to no-reflow. Zhao and colleagues showed that inhibiting the opening of the mitochondrial ATP-sensitive potassium channels prevents simvastatin from exerting a beneficial effect on reducing no-reflow²⁷². Given that the zone of no-reflow is confined within areas of dead cardiomyocytes, and ultrastructural evidence of the microvasculature injury lags behind ultrastructural evidence of cardiomyocyte cell death, no-reflow is unlikely to contribute directly to additional myocyte cell death. However, given that blood-borne trophic factors and cells crucial to the removal of necrotic debris and scar can neither enter nor exit the zone of no-reflow, no-reflow inhibits the healing phase of MI. In our study, we observed that rats subjected to proximal coronary occlusion and reperfusion demonstrated persistent areas of no-reflow at 1 month after acute infarction. Hearts with larger zones of no-reflow and fewer perfused capillaries had greater myocardial infarct expansion (stretching and thinning of the infarcted wall) and dilatation of the left ventricle, signs of poor healing, and adverse LV remodelling²⁷³.

The no-reflow phenomenon has been well documented in patients undergoing reperfusion therapy (either thrombolytic or PCI) for acute STEMI^{268,274–276}. Several different noninvasive imaging techniques, such as echocardiography contrast imaging, nuclear imaging with ²⁰¹Tl-labelled or ^{99m}Tc-labelled albumin microspheres, and MRI with contrast, have been used to document perfusion defects after reperfusion therapy. Preclinical studies verified that MRI-visualized perfusion deficits correlate closely (by location and shape) with perfusion defects observed using thioflavin S staining^{277,278}.

No-reflow has also been associated with adverse LV remodelling in the clinical setting, which is an important observation because the degree of LV remodelling, including dilatation of the LV cavity, is predictive of increased mortality and congestive heart failure after MI^{279–281}. In one study, LV volumes were larger (170 ml) in patients with no-reflow than in patients without no-reflow (115 ml) at 6 months after MI. No-reflow was the most important predictor of LV dilatation, even more so than infarct size, and was the only predictor of cardiac death²⁷⁹. Several clinical studies have also shown that no-reflow in the setting of MI is associated with a poor prognosis^{275,278–280,282,283}. Wu and colleagues demonstrated that patients with no-reflow had more major adverse cardiac events (cardiac death, nonfatal myocardial reinfarction, congestive heart failure, stroke, and unstable angina) over 2 years compared with patients without no-reflow²⁷⁸. This finding was consistent in a multiple regression analysis that accounted for the size of the MI. Ndrepepa and colleagues reported that the presence of no-reflow was predictive of 5-year mortality (OR 1.72–2.01, $P = 0.004$), again independent of infarct size. They concluded that the presence of no-reflow following intervention for STEMI provides prognostic information independent of and beyond that provided by infarct size²⁸².

Several therapies have been targeted at reducing the size of the no-reflow zone. Experimentally, therapies that reduce MI above and beyond reperfusion often reduce the zone of no-reflow within the infarct. In our experience, such therapies must be given before reperfusion to be of use. However, a major issue limiting application of adjunctive therapies is that stopping to administer a drug or therapy before reperfusion is often seen as a delay in door-to-balloon time, and interventional cardiologists are not willing to risk extending that time. We previously described a therapy that reduces no-reflow independent of any effect on the size of the infarct, which can be administered after the infarct artery is patent²⁸⁴. Delayed therapeutic hypothermia administered locally to the risk zone significantly reduced the zone of no-reflow in both rabbit and rat models of MI. In the rabbit model, local hypothermia applied as late as 30 min after establishing patency of the infarct-related artery markedly reduced the size of the no-reflow zone within the infarct²⁸⁴. Myocardial temperature is lowered to 30–34°C for several hours. We believe that this reduction in temperature is likely to reduce endothelial swelling and inflammation, much like how ice reduces blistering after a burn. We plan to determine the long-term consequences of delayed therapeutic hypothermia and predict that it will reduce adverse LV remodelling and long-term cardiac function by enhancing tissue healing. Targeting the no-reflow phenomenon in a way that will not extend door-to-balloon times and is not dependent on a reduction of infarct size provides a novel therapeutic approach that could help to fill the unmet need of unacceptably high morbidity and mortality following MI, despite successful PCI. However, the mechanisms underlying the protective effects of hypothermia on no-reflow are unknown. Based on our observations of the natural history of no-reflow, as well as studies that we and others have conducted in an attempt to understand how early hypothermia can reduce myocardial infarct size, several potential targets of hypothermia have been postulated, including mitogen-activated protein kinases²⁸⁵, local tissue and endothelial oedema, ROS damage and cellular inflammation, and mitochondrial structure and function.

Conclusions

Despite the success of early reperfusion to reduce myocardial infarct size and improve clinical outcome, the high rates of heart failure and mortality following STEMI remain unacceptable. Given that most clinical trials of adjunctive therapies (adjunctive to therapeutic reperfusion) aimed at further reducing myocardial infarct size have been disappointing, new approaches are needed. In this Review, we presented several new therapeutic targets involved in ischaemia–reperfusion injury that have shown promise in preclinical studies, including therapies to reduce myocardial infarct size based on mitochondrial protective properties; approaches that involve the mechanisms of autophagy, mitophagy, and pyroptosis; strategies that take advantage of the cardioprotective properties of certain anaesthetic agents; treatments that show cardioprotective benefits in addition to the effects of certain antiplatelet agents; and therapies that are focused on correcting the no-reflow phenomenon and its effect on healing and adverse LV remodelling.

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Key points

- Mortality after acute ST-segment elevation myocardial infarction (STEMI) has plateaued in the past decade, after a period of continual decline
- Beyond early reperfusion, a variety of adjunctive agents to reduce myocardial infarction after reperfusion have been tested in clinical settings, and have yielded largely disappointing results
- Before clinical testing, candidate cardioprotective agents should be tested in animal models with comorbidities similar to that in patients, and in combination with P2Y₁₂ inhibitors
- Approaches for improving cardiac function following STEMI that have been promising in preclinical studies include those that target mitochondrial bioenergetics, pyroptosis, autophagy, and no-reflow and anaesthetic preconditioning

Figure 1. New and updated approaches to treat the reperfused myocardium

Some of these strategies involve mitochondrial structure and function, whereas others involve anaesthetic preconditioning and reducing the no-reflow phenomenon, which might also involve the mitochondria.

Figure 2. Sites of reactive oxygen species (ROS) production within the mitochondria

Several mitochondrial targets along the outer/inner mitochondrial membranes and within the mitochondrial matrix represent potential approaches to mitigate ischaemia–reperfusion injury. Cyt c, cytochrome c; MAO, monoamine oxidase; NOX-4, NADPH oxidase 4; VDAC, voltage-dependent anion channel.

Figure 3.

The interplay between Toll-like receptor 9 (TLR9) and the NLRP3 inflammasome. TLR9 ligands such as mitochondrial DNA promote production of IL-1 β and IL-18 by activating MyD88 (myeloid differentiation primary response protein 88), which activates the transcription factor nuclear factor- κ B (NF- κ B) through phosphorylation of the NF- κ B inhibitor I κ B. This phosphorylation in turn allows p50 (also known as NF- κ B1) and p65 (also known as NF- κ B p65 subunit) to dimerize, forming active NF- κ B. When triggered by oxidized mitochondrial DNA in the cytosol, NLRP3 (NACHT, LRR, and PYD domains-containing protein 3), the adaptor protein ASC (also known as apoptosis-associated speck-like protein containing a CARD), and pro-caspase 1 form an inflammasome complex that results in the release of active caspase 1. The pro-interleukins are cleaved by caspase 1 into their active form. Caspase 1 also cleaves gasdermin D (GSDMD) to form its N-terminal fragment (GSDMD-nt) that creates holes in the membrane allowing the proinflammatory interleukins and caspase 1 to flow into the interstitial space. Membrane failure caused by those holes also kills the cell, a process termed pyroptosis. Finally, caspase 1 can proteolytically degrade many cytosolic enzymes that can cause further injury. Cyto c, cytochrome c; DeUBase, deubiquitinase; PAMPs, pathogen-associated molecular patterns.

Table 1

MicroRNAs that regulate autophagy in the heart

MicroRNA	Target	Effect on autophagy	Refs
miR-212/132	FOXO3a	Suppression	286
miR-199a	GSK3 β	Suppression	232,236
miR-221	p27	Suppression	236
miR-204	LC3	Suppression	287–289
miR-188-3p	ATG7	Suppression	237
miR-497	LC3b, BCL2	Suppression	233
miR-34a	ATG9	Suppression	290
miR-451	TSC1	Suppression or possibly increased flux	291
miR-30	Beclin 1	Suppression of initiation and/or flux	292
miR-99a	mTOR	Activation	293
miR-144	mTOR	Activation	241