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SPOTLIGHT REVIEW

Calcium-mediated cell death during myocardial reperfusion

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Abstract Reperfusion may induce additional cell death in patients with acute myocardial infarction receiving primary angioplasty or thrombolysis. Altered intracellular Ca^{2+} handling was initially considered an essential mechanism of reperfusion-induced cardiomyocyte death. However, more recent studies have demonstrated the importance of Ca^{2+}-independent mechanisms that converge on mitochondrial permeability transition (MPT) and are shared by cardiomyocytes and other cell types. This article analyses the importance of Ca^{2+}-dependent cell death in light of these new observations. Altered Ca^{2+} handling includes increased cytosolic Ca^{2+} levels, leading to activation of calpain-mediated proteolysis and sarcoplasmic reticulum-driven oscillations; this can induce hypercontracture, but also MPT due to the privileged Ca^{2+} transfer between sarcoplasmic reticulum and mitochondria through cytosolic Ca^{2+} microdomains. In the opposite direction, permeability transition can worsen altered Ca^{2+} handling and favour hypercontracture. Ca^{2+} appears to play an important role in cell death during the initial minutes of reperfusion, particularly after brief periods of ischaemia. Developing effective and safe treatments to prevent Ca^{2+}-mediated cardiomyocyte death in patients with transient ischaemia, by targeting Ca^{2+} influx, intracellular Ca^{2+} handling, or Ca^{2+}-induced cell death effectors, is an unmet challenge with important therapeutic implications and large potential clinical impact.

Keywords Myocardial infarction • Reperfusion injury • Calpain • Mitochondria

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1. Background: from Ca^{2+} paradox to oxygen paradox

Myocardial reperfusion injury, defined as a kind of cell death occurring secondary to transient ischaemia that is preventable by interventions applied at the time of reperfusion,[1–6] is now recognized as an important element of the pathophysiology of myocardial ischaemia–reperfusion. However, its cellular and molecular mechanisms are far from being completely elucidated.[3] Alterations in cellular Ca^{2+} homeostasis have been considered part of these mechanisms during the past few decades, and several hypotheses have been proposed to explain their causative role in cell death.

Electron microscopy studies performed at different times after coronary reperfusion demonstrated rapid and massive Ca^{2+} deposition in the mitochondrial matrix of reperfused myocardium.[7,8] The initial hypotheses on the mechanism of reperfusion injury conceded a capital importance to cytosolic Ca^{2+} overload. In fact, the so-called Ca^{2+} paradox (hypercontracture and death of cardiomyocytes occurring upon restoration of Ca^{2+} after a Ca^{2+}-free perfusion period) was meant to be a first cellular model of reperfusion injury.[9] Hypercontracture was recognized as the cause of the characteristic histological appearance of reperfused infarcts known as contraction band necrosis.[10] Hypercontracture could also be observed in isolated perfused hearts submitted to reoxygenation after transient hypoxia, a phenomenon that was named 'oxygen paradox'.[11] Hypercontracture was proposed to be a triggering cause of cell death in isolated cardiomyocytes[12] and intact tissue,[13] as contractile blockers administered at the time of reperfusion were able to prevent it.

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Subsequent studies identified mitochondrial permeability transition (MPT) as an important cause of reperfusion injury.[14–16] Although MPT was originally described as a consequence of increased mitochondrial Ca^{2+} load,[17] it was soon realized that other triggers were also involved. The role of Ca^{2+} as a cause of MPT lost ground in favour of reactive oxygen species (ROS), and Ca^{2+} is nowadays more often seen to play a secondary role in the pathogenesis of reperfusion injury.[18–20] A prominent recent review on reperfusion injury (covering not only myocardium) contains no reference to Ca^{2+} at all.[6] In the present article, we revisit the role of Ca^{2+} in reperfusion-induced cardiomyocyte death in the light of recently available information.

2. Alterations of Ca^{2+} handling during ischaemia

2.1 Na^+ and Ca^{2+} overload during ischaemia

Anaerobic glycolysis and H^+ released by ATP breakdown produce a progressive decline in intracellular pH (pHi) during the initial minutes of ischaemia.[21] ^{31}P-NMR spectroscopy studies demonstrated pHi values of about 6.4 after 20 min of ischaemia in isolated rat hearts.[22] These initial changes are associated with a sustained rise in intracellular Na^+ as determined by ^{23}Na-NMR spectroscopy.[23,24] This increase in intracellular Na^+ has been attributed to a decrease in the energy-dependent Na^+ extrusion due to the inhibition of Na^+/K^+-ATPase, and to an increased Na^+ influx associated with the activation of H^+ extrusion mechanisms by Na^+/H^+-exchanger (NHE) and Na^+-HCO_3 cotransporters,[25–27] but also to persistent (non-inactivating) Na^+ channels.[28,29] The relative contribution of NHE and persistent Na^+ channels has not been satisfactorily resolved.[28] The fact that NHE activity is reduced by low extracellular pH as occurs during ischaemia,[30] and that the use of the first non-selective NHE inhibitors, such as amiloride, also inhibited persistent Na^+ channels,[28] has resulted in the role of NHE in the rise in intracellular Na^+ levels being questioned. In this sense, initial studies by Xiao and Allen argued that NHE1 is substantially inhibited during ischaemia and that it becomes activated during early reperfusion.[31,32] According to these authors, activation during this phase would be critical for reperfusion injury. However, more recent studies using a new generation of NHE inhibitors[33,34] and mice with null mutation in the NHE1 gene[35] have shown convincingly a contribution of NHE to cytosolic Na^+ overload during ischaemia.

Studies in isolated cardiomyocytes labelled with fluorescence markers of Ca^{2+}[36,37] or isolated perfused hearts using surface fluorescence[38] or 19F-NMR spectroscopy[39] have demonstrated a sustained rise in Ca^{2+} during ischaemia, which starts after the Na^+ rise in close temporal association with rigor onset. When the ATP concentration reaches a critically low threshold (below 100 μmol/L), a force-generating crossbridge cycling is initiated, and rigor contracture develops.[40] Rigor contracture at this low ATP concentration is essentially Ca^{2+} independent,[40,41] but its onset marks the beginning of the cytosolic Ca^{2+} rise and represents a key event in the progression of ischaemic injury.[42,43] These low ATP concentrations would impair the activity of sarcolemmal Na^+/K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), thus acting as a trigger for both cytosolic Na^+ and Ca^{2+} overload.

The contribution of sarcolemmal NCX to cytosolic Ca^{2+} overload during ischaemia has been consistently reported in studies involving pharmacological inhibition of NCX, its genetic ablation, or reduced expression via adenovirally delivered shRNA.[39,44–47] The direction of NCX operation depends on the difference between transmembrane potential and the reversal potential of NCX, and is therefore determined by the intra- and extracellular concentrations of Na^+ and Ca^{2+}.[48] Under physiological conditions, the exchanger operates mainly in its forward mode to extrude Ca^{2+} from the cytosol during diastole,[49] but membrane depolarization and a reduced Na^+ gradient, as a consequence of the intracellular Na^+ rise, determine a net Ca^{2+} influx through reverse NCX transport during ischaemia. However, the fact that prevention of Na^+ overload only attenuates the rise in cytosolic Ca^{2+} during ischaemia[25] suggests a contribution of additional, Na^+-independent ways of Ca^{2+} entry, e.g. via the L-type Ca^{2+} channel.[50]

Under normal conditions, the concentration of Ca^{2+} in the mitochondrial matrix is very low and similar to that in cytosol.[51] Ca^{2+} enters the matrix mainly through the Ca^{2+} uniporter, dependent on the transmembrane electrochemical gradient, and is mainly extruded via the mitochondrial NCX, which is influenced by the matrix Na^+ concentration[52] and which, in turn, depends on the H^+ concentration and the activity of mitochondrial NHE.[53] The H^+ gradient across the mitochondrial membrane, built up by respiration and dissipated by ATP synthesis, is modulated by different ion transporters and exchangers, prominently the KHE.[54] During myocardial ischaemia, the rise in cytosolic Ca^{2+} levels tends to increase mitochondrial Ca^{2+} influx, but dissipation of the transmembrane potential has the opposite effect. However, dissipation of the mitochondrial membrane potential is incomplete during a prolonged ischaemic period. Thus, it is generally admitted that mitochondrial Ca^{2+} influx is important during initial ischaemia and that it remains increased thereafter.[55,56]

2.2 Consequences of Ca^{2+} overload during ischaemia

2.2.1 Cell-to-cell uncoupling

In the ischaemic cardiomyocyte, the onset of the rise of cytosolic Ca^{2+} is closely followed by cell-to-cell electrical uncoupling.[43,57] It is still unclear whether the effects of Ca^{2+} are direct or mediated through intracellular messengers. Ca^{2+} probably induces connexin43 (Cx43) gap junctional closure by activation of calmodulin, which may act directly as a gating particle.[57] pHi is also an important regulator of Cx43 gap junction channel permeability, and acidosis probably contributes to uncoupling.[57] Closure of gap junctional channels by intracellular Ca^{2+} during ischaemia may protect cells from membrane depolarization and leakage of metabolites through gap junctions by disconnecting them from damaged cells.[58] However, reduced cell coupling may cause arrhythmias.[59]

2.2.2 Opening of connexin hemichannels

Both extracellular and intracellular Ca^{2+} concentrations modulate unopposed connexin hemichannel gating. A decrease in extracellular Ca^{2+} has been shown to induce hemichannel opening[60,61] and increase Cx43 hemichannel pore diameter.[62] Intracellular Ca^{2+} also regulates hemichannel function in a biphasic manner: moderate increases in Ca^{2+} can induce Cx43 hemichannel opening, whereas larger increases in Ca^{2+} inhibits hemichannel activity.[63] The opening of Cx43 hemichannels triggered by moderate intracellular Ca^{2+} levels has been suggested to involve multiple intermediate signalling

steps[64] and intramolecular Cx43 loop/tail interactions.[63] Ischaemia induces opening of connexin hemichannels, which may contribute to cell injury and arrhythmias[65] through deleterious Ca^{2+} influx.[66] Hemichannel opening induces release of intracellular metabolites, such as ATP, inositol 1,4,5-triphosphate (IP_3), cAMP, NAD^+, or glutamate, to the extracellular space[67,68] that may be involved in paracrine cardioprotective signalling.[68,69]

2.2.3 Calpain translocation to the cell membrane

Calpains represent a wide family of non-lysosomal, Ca^{2+}-dependent thiol proteases implicated in basic cellular processes including differentiation, proliferation, and cell migration and are tightly regulated by the cellular control of Ca^{2+} and its endogenous inhibitor calpastatin. Loss of Ca^{2+} homeostasis results in an unregulated overactivation of calpain.[70] The contribution of calpains to ischaemia/reperfusion injury has been consistently reported by several groups,[71–74] and the kinetics of calpain activation have been recently determined.[75] It has been proposed that under physiological conditions, translocation of calpains to the sarcolemma in response to transient cytosolic Ca^{2+} elevation is an obligatory step in the process of their activation.[76] However, translocation seems not to be essential under situations leading to a dysregulation of intracellular Ca^{2+} levels.[75] *In vitro* studies have shown that calpain activity is highly influenced by pHi.[77] Recent results from our group demonstrated that intracellular acidosis prevents calpain activation during ischaemia, despite high intracellular Ca^{2+} concentrations.[75]

2.2.4 Mitochondrial Ca^{2+} overload

It is generally assumed that mitochondrial Ca^{2+} accumulation has detrimental cellular effects, facilitating mitochondrial membrane permeabilization and energetic collapse. Prevention of mitochondrial Ca^{2+} uptake has a protective effect in ischaemia–reperfusion injury.[78,79] However, normoxic healthy mitochondria exhibit a striking ability to accumulate enormous amounts of Ca^{2+} and efficiently participate in cellular Ca^{2+} buffering along with the sarcoplasmic reticulum (SR).[80] Mitochondrial Ca^{2+} uptake and storage capacity may be particularly relevant when cytosolic Ca^{2+} is elevated, as during ischaemia. Nevertheless, the concept that mitochondria can shape the spatiotemporal pattern of cytosolic Ca^{2+} during ischaemia has been controversial. While mitochondrial membrane depolarization is expected to preclude Ca^{2+} uptake through the mitochondrial Ca^{2+} uniporter,[81] significant mitochondrial Ca^{2+} accumulation has been documented during ischaemia in the presence of residual mitochondrial membrane potential.[55,56] Moreover, pharmacological inhibition of Ca^{2+} uptake during ischaemia has been shown to have a pernicious effect on cell survival upon re-energization, an effect that is associated with an impairment of cytosolic Ca^{2+} handling,[55] supporting the concept that mitochondrial Ca^{2+} uptake helps to delay a rise in cytosolic Ca^{2+} levels.

3. Intracellular Ca^{2+} handling during reperfusion

The main mechanisms and consequences of altered Ca^{2+} handling during reperfusion are summarized schematically in *Figure 1*.

3.1 Na^+ and Ca^{2+} influx during initial reperfusion

The intracellular Na^+ overload observed in reperfused cardiomyocytes is largely the consequence of Na^+ gain during prior ischaemia,[23,24] but also of additional Na^+ influx at the onset of reperfusion associated with pHi correction or Na^+ influx from adjacent myocytes via gap junctions.[82]

Reperfusion removes extracellular H^+ and rapidly reactivates intracellular H^+ efflux mediated by the lactate-H^+ cotransporter[83] and the activity of NHE and Na^+HCO_3 cotransporter,[83] resulting in a fast pHi recovery.[22 23] Na-NMR studies demonstrate a fast correction in intracellular Na^+ levels during initial reperfusion even in the presence of NHE inhibitors,[84] which can probably be explained by the compensatory action of bicarbonate transporters and the reactivation of Na^+/K^+-ATPase.[22,85,86]

Reactivation of Na^+/K^+-ATPase appears to be a key determinant of the kinetics of Na^+ recovery at reperfusion.[87] Whereas after short periods of ischaemia functionality of the Na^+/K^+-ATPase is preserved immediately upon reperfusion,[86,88] it is impaired after prolonged occlusions.[88,89] It has been proposed that oxidation of sulfhydryl groups on the protein,[90] and more recently, calpain-dependent loosening of its alpha subunit from the membrane–cytoskeleton complex,[88] contribute to reperfusion-induced Na^+/K^+-ATPase dysfunction. During the first minutes of reperfusion, cytosolic Na^+ overload provides a large driving force for Ca^{2+} influx through the reverse mode of NCX. Inhibitors of NCX applied at the time of reperfusion reduce the frequency of Ca^{2+} oscillations (see below), infarct size, and myocardial stunning in intact hearts, confirming that early reperfusion results in an additional Ca^{2+} influx through the reverse-mode operation of NCX.[5,91,92]

3.2 SR-driven Ca^{2+}oscillations and hypercontracture

The SR is the main intracellular Ca^{2+} store in cardiac myocytes, orchestrating excitation–contraction coupling through rapid Ca^{2+} uptake (SERCA ATPase) and release transport system (ryanodine receptor, RyR) facilitated by a close juxtaposition with T-tubules and mitochondria.[93] Previous studies have indicated that in the presence of an abnormally high cytosolic Ca^{2+} concentration, such as that occurring in the very first minutes of reperfusion, a rapid SR Ca^{2+} cycling may be the genuine cause of reperfusion-induced hypercontracture.[94,95] Ca^{2+} oscillations are the consequence of the reactivation of mitochondrial respiration and efficient ATP transfer to the SR, subsequent Ca^{2+} uptake by SERCA ATPase, and Ca^{2+} release through RyR when SR storage capacity is exceeded.[94] Importantly, upon resumption of mitochondrial energy synthesis, SERCA ATPase may initiate this futile Ca^{2+} cycling before other ATP-dependent sarcolemmal Ca^{2+} extruders reduce cytosolic Ca^{2+} load, in part due to the privileged spatial communication between SR and mitochondria (see below). Ca^{2+} release from a SR unit may be taken up by an adjacent one, and the resulting Ca^{2+} oscillation can propagate throughout the cell as Ca^{2+} waves that induce hypercontracture. Accordingly, a decrease in the synthesis of ATP necessary to activate SERCA ATPase,[96] pharmacological inhibition of SERCA or RyR,[94,97,98] or a reduction in the cytosolic Ca^{2+} load[5,99] decreases the frequency and amplitude of SR-induced Ca^{2+} sparks and oscillations and reduces hypercontracture and cell death in different experimental models. Alternatively, the improvement of SR Ca^{2+}

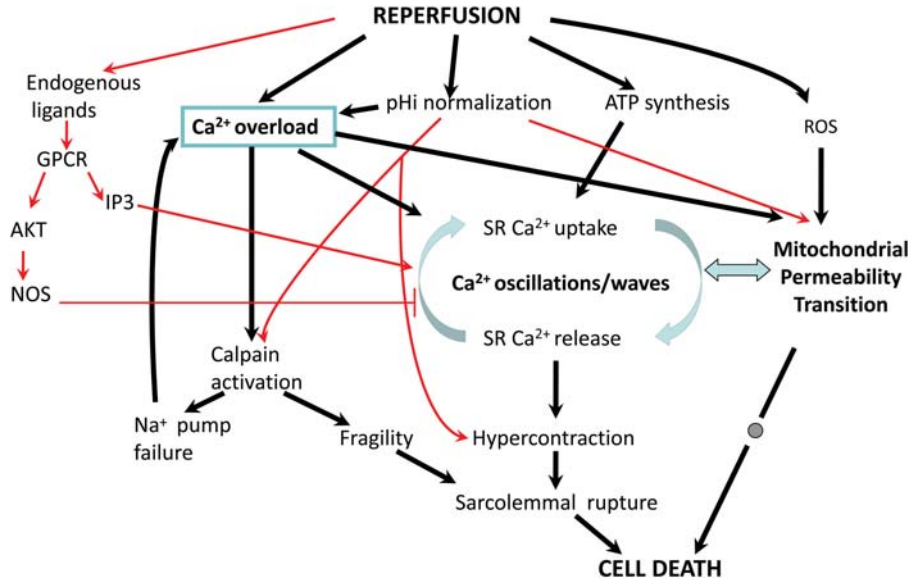

Figure 1 Mechanisms and consequences of altered Ca^{2+} handling in cardiomyocytes during initial reperfusion. Main events are connected through black lines, whereas red lines indicate important modulating factors. GCPR, G-coupled protein receptors; IP3, inositol trisphosphate; NOS, nitric oxide synthase; ROS, reactive oxygen species.

sequestration capacity by protein kinase G (PKG)-dependent phosphorylation of phospholamban (resulting in more efficient SERCA activity) may decrease reperfusion-induced SR Ca^{2+} oscillations, favour the recovery of cytosolic Ca^{2+} control, and prevent excessive myofibrilar activation during reperfusion.[95,100] These results are in agreement with recent observations indicating that Ca^{2+} wave development depends on the balance between SERCA-dependent Ca^{2+} reuptake and threshold SR Ca^{2+} content[101] and underline the importance of energy-dependent SR-driven Ca^{2+} oscillations in the pathophysiology of ischaemia–reperfusion.

3.3 Role of mitochondria–SR interaction in hypercontracture and MPT

Two subpopulations of mitochondria, subsarcolemmal (SSM) and interfibrillar (IFM), have been described,[102] with differences in morphology,[103] functional capacity,[102,104] and, more importantly, differential interactions with other cellular components, as only IFM is in close contact with the SR. Mitochondria and SR are structurally and functionally interconnected, and this interplay has important pathophysiological consequences that go beyond the individual role of each organelle.[105,106] It has been demonstrated that SR Ca^{2+} uptake may be altered when creatine kinase activity from IFM is specifically depressed.[107] As a consequence, the highly specialized energy transfer and Ca^{2+} exchange system formed by SR, IFM, and myofilaments becomes less efficient and may account for the mismatch between energy demand/utilization and Ca^{2+} uptake/release (contractile force) that is observed under certain pathological conditions, such as heart failure.[107] During reperfusion, microdomains of high Ca^{2+} concentration around RyR of the SR have been proposed to be involved in the permeabilization of mitochondria located in close proximity.[97,98] Accordingly, pharmacological blockade of SR Ca^{2+} load with thapsigargin/ryanodine impacted on mitochondrial Ca^{2+} uptake kinetics in intact cardiac myocytes, but had no effect in isolated mitochondria, where the contribution of SR is expected to be negligible.[98] The distance between SR and mitochondria has been estimated to be 10–50 nm,[108] and mitofusin 2 has been identified as the main mediator of the physical link among the two organelles.[109] Chemical disruption of the microtubule network increases the inter-organelle distance and reduces the noxious effect that the SR-induced Ca^{2+} oscillations exert on mitochondrial integrity during the first minutes of reperfusion.[98] The differences in Ca^{2+} crosstalk with SR may explain in part why IFM have a higher Ca^{2+} retention capacity,[102] and this difference in Ca^{2+} tolerance may exacerbate cellular stress during reperfusion, when damaged mitochondria (releasing Ca^{2+} as well as other molecules) coexist along with intact mitochondria (capable of sustaining ATP synthesis) within the same cell. Moreover, the relationship between SR and mitochondria appears to be bidirectional: SR-induced Ca^{2+} cycling may favour mitochondrial permeabilization and energetic collapse on one hand,[97,98,110] whereas mitochondrial permeabilization may trigger SR Ca^{2+} oscillations, hypercontracture, and cell death during reperfusion by increasing cytosolic Ca^{2+} overload[111] on the other hand. Thus, early pharmacologic inhibition of SR Ca^{2+} uptake and release upon re-energization reduces MPT, hypercontracture, and cell death,[98] and genetic or chemical blockade of mitochondrial permeabilization decreases hypercontracture and infarct size.[112]

3.4 Normalization of intracellular pH

A rapid correction of acidosis during reperfusion contributes to Ca^{2+} influx and, furthermore, may precipitate the adverse effects of Ca^{2+}

overload that remain inhibited at low pHi. Normalization of pHi allows the activation of critical proteins involved in the regulation of Ca^{2+} handling, including NCX,[22,113] the L-type Ca^{2+} channel,[114] the RyR,[115] and SERCA.[116] Even a brief prolongation of extracellular acidosis during the early phase of reoxygenation reduces Ca^{2+} overload.[117] Recovery from pHi also relieves the inhibition of cardiomyocyte contractility caused by a reduced Ca^{2+} binding to troponin C,[118] the occurrence of MPT,[119,120] activation of calpains,[75] and blockade of gap junctions.[121] Therefore, the relative timing of the correction of intracellular Ca^{2+} levels and pHi during the first minutes of reperfusion has been proposed to determine cell death (recovery of pHi occurs before that of Ca^{2+}) or survival (recovery of Ca^{2+} control occurs before pHi normalization). This hypothesis may help explain the cardioprotective effect of brief acidic reperfusion and ischaemic postconditioning.[122]

3.5 Extracellular signalling modulating intracellular Ca^{2+}

The intracellular Ca^{2+} concentration can be modulated by a number of extracellular signals during reperfusion. Activation of membrane-bound guanylyl cyclase with natriuretic peptides or soluble guanylyl cyclase with NO donors increases the cGMP synthesis, which in turn activates PKG. PKG has been shown to activate SERCA through phosphorylation of phospholamban, increasing Ca^{2+} accumulation by the SR and preventing cytosolic Ca^{2+} oscillations.[123]

G protein-coupled receptors (GPCRs) might also play a relevant role under ischaemia–reperfusion in modulating intracellular Ca^{2+} levels through two opposing mechanisms. Activation of $G_{\alpha q/11}$ by most GPCRs results in the hydrolysis of membrane-bound phosphatidylinositol 4,5-bisphosphate (PIP2) by phospholipase Cβ, releasing inositol 1,2,5-trisphosphate (IP3). This molecule binds to its intracellular receptors (IP3R), inducing the release of Ca^{2+} from the endoplasmic reticulum to the cytosol.[124] On the other hand, binding of a ligand to its GPCR also leads, probably through the βγ subunits of the G proteins, to serial activation of phosphoinositide 3-kinase (PI3K), Akt, ERK, nitric oxide synthase (NOS), and guanylyl cyclase, which results in increased levels of intracellular cGMP, activation of SERCA through PKG-mediated phosphorylation of phospholamban, and uptake of Ca^{2+} by the SR.[123] Adenosine, ATP (P2Y receptors), opioids, bradykinin, adrenaline, acetylcholine, insulin, erythropoietin, oestrogens, transforming growth factor-β1, angiotensin II, and adrenomedullin are endogenous ligands that may modulate intracellular Ca^{2+} by binding to GPCR. Most of them have been shown to activate the PI3K/Akt/ERK/NOS signalling pathway and to be involved in cardioprotection by pre- and postconditioning.[125] Similarly, extracellular Ca^{2+}-sensing receptors are GPCR that can play a role in regulation of the intracellular Ca^{2+} concentration and have been shown to be involved in ischaemia/reperfusion-induced apoptosis in rat cardiomyocytes by increasing the intracellular Ca^{2+} concentration.[126] On the other hand, Ca^{2+}-sensing receptors have been suggested to play a role in preconditioning protection in isolated mouse hearts.[127] Protease-activated receptors, such as thrombin receptors, are specialized GPCR. Thrombin activates its receptor by cleaving part of its extracellular amino-terminal domain, promoting coupling with $G_{\alpha\beta\gamma}$ proteins, and modulating the intracellular Ca^{2+} concentration. Thrombin has been shown to increase cytosolic Ca^{2+} and twitch amplitude in isolated rat cardiomyocytes[128] and to cause cell death after ischaemia–reperfusion.[129]

4. The end-effectors of Ca^{2+}-dependent cardiomyocyte death during reperfusion

Altered Ca^{2+} handling may trigger processes that directly cause cell death such as hypercontracture, proteolysis, and MPT.

4.1 Hypercontracture

The role of mechanical stress generated by an excessive contractile activation, known as hypercontracture, in the development of cardiomyocyte death during reperfusion is supported by ample experimental evidence.[10,130,131] Hypercontracture has been observed during the first minutes of reperfusion *in vitro* by microscopic techniques, and *in vivo* by intramyocardial ultrasonometry.[130,132] There is a close correlation between the magnitude and time course of hypercontracture and enzyme release in reperfused myocardium.[112,130] Brief contractile inhibition at the onset of reperfusion prevents enzyme release for the time the inhibition is present, while prolonged contractile inhibition limits final infarct size.[88,133,134]

Hypercontracture is induced by recovery of energy production in the presence of a high cytosolic Ca^{2+} concentration[132] (*Figure 2*). Cytosolic Ca^{2+} oscillations lead to sustained and uncontrolled activation of the contractile apparatus that results in hypercontracture.[131,135] Isolated, unrestrained cardiomyocytes, but not those restrained by attachment to a micropipette,[136] can maintain sarcolemmal and metabolic integrity after hypercontracture.[12,137] However, in tissue, the mechanical forces resulting from hypercontraction of adjacent cells lead to mutual cellular disruption and necrosis.[138,139]

NMR spectroscopy shows rapid and virtually complete but transient energy recovery in hearts undergoing hypercontracture and important necrosis after transient ischaemia, demonstrating that hypercontracture is not due to low ATP levels in those experiments.[112] Furthermore, contractile blockade prevents cell death (LDH release) and cell de-energization, indicating that the fall in energy is not a cause but a consequence of hypercontracture.

It has been proposed that rigor-type, Ca^{2+}-independent activation of the contractile machinery contributes to hypercontracture, at least under certain conditions.[140] Rigor-type contracture may be activated during reoxygenation if re-energization of the ischaemic cardiomyocytes occurs at a very low rate as after prolonged or severe ischaemia.[40,141] It is unclear, however, under which pathophysiological conditions *in vivo* this state of very slow, but non-zero re-energization required for rigor-type contracture may actually prevail. It is not expected to occur when ATP levels are rapidly restored after brief ischaemia or when reactivation of mitochondrial respiration completely fails or is effectively suppressed.[142,143]

4.2 Calpain-mediated proteolysis

During reperfusion calpains hydrolyze proteins from the sarcolemma and the cytoskeleton, including α-fodrin and ankyrin. α-Fodrin forms the backbone of the membrane cytoskeleton. Its degradation correlates with increased fragility of the membrane, reducing the tolerance of the sarcolemma to the mechanical stress associated with hypercontracture and acute cell swelling during reperfusion.[71,74,144] 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.[145] Binding to ankyrin connects Na^+/K^+-ATPase to the fodrin-based membrane cytoskeleton and determines its

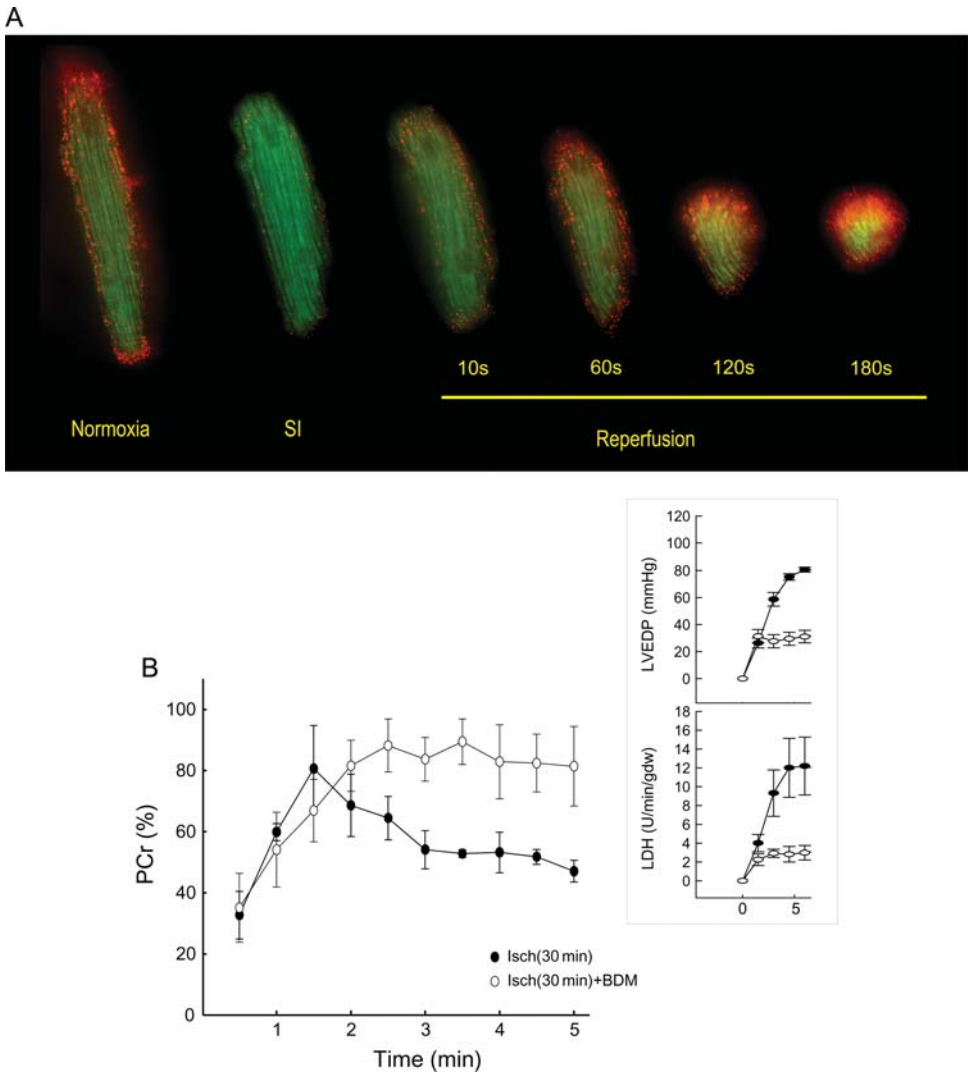

Figure 2 (*A*) Calcium and energy-dependent hypercontracture. Simulated ischaemia (SI, exposure to hypoxia at pH 6.4) and reperfusion in myocytes from adult rat heart labelled with JC-1 (5′,6,6′-tetrachloro-1,1′,3,3′- tetraethylbenzimidazolylcarbocyanine iodide) to study mitochondrial membrane potential. Ischaemia causes loss of membrane potential (disappearance of the red signal) and rigour shortening. Reperfusion causes recovery of mitochondrial membrane potential and hypercontracture. (From ref. 112; used with permission). (*B*) Analysis of recovery of cell energy (% of phosphocreatine, PCr) by NMR spectroscopy during the initial minutes of reperfusion in rat hearts submitted to 30 min of ischaemia. There is an initial recovery followed by de-energization that is prevented by contractile blockade. The inset shows that de-energization occurring in the control group is coincident with the development of hypercontracture that is manifested as an increase in left ventricular end-diastolic pressure (LVEDP) with lactic dehydrogenase (LDH) release reflecting sarcolemmal rupture, both prevented by contractile blockade with BDM. Altogether, these results indicate that de-energization is a consequence of hypercontracture-mediated cell death and not a cause of it. (Modified from ref. 112; with permission).

specific localization in the membrane and its correct function.[146] 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. This results in impaired normalization of the cytosolic Na^+ concentration and in further Ca^{2+} influx via reverse-mode NCX.[88] It has been proposed that calpain can also modulate Ca^{2+} handling by cleaving RyR[147] and SERCA2a.[148] In addition, calpain-dependent activation of Bid has been described to induce the release of cytochrome c and other proapoptotic factors.[149] Calpain inhibition reduces infarct size in different models[72,75] and has been proposed to be an important element in the cardioprotective effect of pre- and postconditioning.[23,71]

4.3 Mitochondrial permeability transition

Upon reperfusion, cytosolic Ca^{2+} overload in the presence of mitochondrial repolarization (although incomplete) provides the electrochemical force necessary to activate mitochondrial Ca^{2+} uptake through the Ca^{2+} uniporter. However, cumulative Ca^{2+} capacity of reperfused mitochondria is severely impaired, mainly as a consequence of the concurrence of oxidative stress and low cellular ATP concentration, conditions that may initiate mitochondrial failure upon Ca^{2+} uptake when intracellular pH is normalized.[14–16]

Mitochondrial failure occurs as a consequence of the abrupt increase in the permeability of mitochondrial membranes that are incompatible with efficient respiration and ATP synthesis. This phenomenon, known as mitochondrial membrane permeabilization, was initially described *in vitro* as a response specifically induced by Ca^{2+}.[17] It appears to be mediated by cyclophilin D (CyD), a trans-isomerase protein located at the inner mitochondrial membrane, although other indirect mechanisms of mitochondrial permeabilization have been described.[150,151] Genetic ablation of CyD or its pharmacological inhibition by CsA or other drugs increases mitochondrial tolerance to Ca^{2+} overload and reduces cell death after transient ischaemia in different experimental models.[152–154] In fact, tolerance to external Ca^{2+} pulses of mitochondria isolated from reperfused myocardium has been used as an index of the susceptibility of the myocardium to MPT under different conditions, including cardioprotective interventions.[152] However, the causal role of mitochondrial Ca^{2+} tolerance as a mechanism of attenuated MPT during *in vivo* reperfusion remains obscure, and other triggers, in particular ROS, have been demonstrated to be more important in intact myocardium.[155] Indeed, the Ca^{2+} threshold upon which isolated mitochondria experience acute failure *in vitro* (high micromolar range) could only be achieved *in vivo* as the consequence of sarcolemmal disruption, resulting in massive Ca^{2+} entry from the extracellular space. However, studies using engineering-based fluorescent techniques have demonstrated that Ca^{2+} concentrations can be dramatically increased in specific microanatomical domains, reaching levels that are well above the concentration achieved in the bulk of cytoplasm,[156] and more recent evidence indicates that these microdomains may explain the occurrence of reperfusion-induced mitochondrial permeabilization in some cellular regions.[97,98] Moreover, the possibility that a subpopulation of intact, energy-producing mitochondria coexist with severely damaged mitochondria undergoing membrane permeabilization has been proposed as the mechanism reconciling both ATP-dependent hypercontracture and mitochondrial failure as the cause of reperfusion-induced cell death, because membrane permeabilization of mitochondria previously overloaded with Ca^{2+} may further impair cytosolic Ca^{2+} handling.[111]

4.4 Relative importance of Ca^{2+}-driven hypercontracture and MPT as causes of cell death

Occurrence of MPT in a subset of mitochondria releases Ca^{2+} into the cytosol, favouring Ca^{2+} oscillations/overload that eventually lead to hypercontracture. Conversely, uncontrolled SR-triggered Ca^{2+} oscillations may induce MPT in mitochondria located in close proximity to the SR. In addition, it is also possible that both mechanisms of cell death operate separately: altered Ca^{2+} handling can cause cell death through calpain-mediated proteolysis and hypercontracture independently of MPT, whereas MPT can be the primary cause of cell death during reperfusion. In fact, reperfusion-induced cell death occurs in cells other than cardiomyocytes that lack significant contractile machinery, such as hepatocytes, neurons, or modified cardiomyocyte cell lines.[120,157] The question of the relationship and relative importance of these different cell death mechanisms during myocardial reperfusion is thus relevant. We recently proposed that the relative contribution of MPT to cell death depends on the duration of prior ischaemia. After brief periods of ischaemia, inhibition of mitochondrial permeabilization with CsA or genetic ablation of CyD failed to protect isolated cardiomyocytes or intact hearts from reperfusion injury, whereas prevention of Ca^{2+}-induced, energy-dependent hypercontracture was effective. Conversely, after more prolonged ischaemia, inhibition of MPT was strongly protective.[112]

4.5 Role of apoptosis

Ca^{2+} overload is an established trigger of apoptosis, and it has been proposed that it can induce apoptosis of cardiomyocytes even when its magnitude is lower than that required to induce necrosis, as may occur, for example, during inotropic stimulation.[158] It is thus theoretically possible that Ca^{2+} overload can induce apoptotic cell death in cardiomyocytes surviving the initial minutes of reperfusion. There are, however, reasons to believe that this is not a relevant cause of cell death. Most studies agree that infarct size, determined early during reperfusion, does not increase significantly during the following hours.[159,160] Furthermore, there is solid evidence that the caspase pathway responsible for mitochondria-driven apoptosis is not operative in adult cardiomyocytes.[161]

5. Pharmacological targets against Ca^{2+}-mediated reperfusion injury

5.1 Improving Ca^{2+} handling

The value of cell systems involved in exaggerated Ca^{2+} influx during reperfusion as pharmacological targets to be used against reperfusion injury has been discussed in previous sections and in other articles.[162] We will focus here on the potential value of systems involved in intracellular Ca^{2+} handling as therapeutic targets.

SERCA activity is specifically regulated by phospholamban (PLB), which in its dephosphorylated form exerts an inhibitory effect, susceptible to be relieved after its phosphorylation by either cAMP- or cGMP-dependent protein kinases or the Ca^{2+}-calmodulin-dependent protein kinase (CaMKII).[163] Because ischaemia induces dephosphorylation of PLB,[164] modulation of the phosphorylation status of PLB has attracted much attention as a potential therapeutic strategy to reduce reperfusion injury. Calcineurin inhibitors (e.g. CsA) have been demonstrated to prevent PLB dephosphorylation by inhibiting PKC-α translocation.[164] Hsp20 has been recently identified as a modulator of PLB phosphorylation,[165] but its potential value as a pharmacological target remains to be established. Natriuretic peptides increase PLB phosphorylation via PKG and are cardioprotective.[166] B-type natriuretic peptide also inhibits the mitochondrial Ca^{2+} uniporter, reduces ROS generation, and improves mitochondrial energy recovery.[167] It may seem paradoxical that an increase in CaMKII activity has been found to decrease cell viability in rat hearts and isolated myocytes subjected to transient ischaemia–reperfusion, an effect that could be reverted in the presence of the CaMKII inhibitor KN-93 or the CaMKII inhibitory peptide AIP[168]: the adverse effect of CaMKII may, however, be due to its effect on targets other than PLB.

Regulation of the crosstalk between SR and mitochondria is emerging as a new target to limit cell death. A recent report indicates that postconditioning may protect cardiomyocytes from apoptotic death induced by transient ischaemia by preserving crosstalk between mitochondria and the SR.[169] In some cases, drugs known for their effects on sarcolemmal ionic exchangers have been shown to inhibit mitochondrial transporters. It has been suggested that part of the protective effect of cariporide against ischaemic damage is due to its inhibitory effect on mitochondrial NHE exchange that delays $\Delta\Psi m$

dissipation and Ca^{2+} disturbances associated with energy depletion.[53] Also, the plasma membrane NCX inhibitor KB-R7943 may inhibit the mitochondrial Ca^{2+} uniporter and prevent mitochondrial Ca^{2+} overload during ischaemia–reperfusion in addition to its effect on cytosolic Ca^{2+} levels.[170]

5.2 Attenuating the consequences of Ca^{2+} overload

A major limitation of strategies aimed towards limiting Ca^{2+} overload during reperfusion is that they cannot prevent its accumulation occurring during preceding ischaemia. An alternative approach is to inhibit Ca^{2+}-dependent effects of reperfusion injury.

Contractile inhibitors prevent enzyme release during initial reperfusion[88,133] and limit infarct size.[134,171] The drug most extensively used for this purpose has been 2,3-butanedione monoxime (BDM), a reversible blocker of actomyosin-ATPase. Recently, a new contractile inhibitor, blebbistatin, has been shown to inhibit contractility with a potency that is three orders of magnitude higher.[172] The dramatic effect blebbistatin has on the reduction of infarct size when administered to isolated rat hearts (unpublished results from our group) makes this drug a potential candidate for translation.

The cardioprotective effects obtained with some strategies aimed at preventing mechanisms other than hypercontracture could be explained, at least in part, by their actions on contractility. Delay in pHi recovery induced by pharmacological treatments,[22,85] transient reperfusion with respiratory or metabolic acidosis[4,22] or postconditioning[23] inhibits myofibrillar contractility during initial reperfusion. Similarly, cardioprotection afforded by stimulation of the cGMP/PKG pathway[100,173,174] has been related to PKG-dependent effects on the sensitivity of myofibrils to Ca^{2+}.[175,176] Moreover, activation of PKG may delay pHi normalization through inhibition of NHE.[177]

The fact that Ca^{2+}-dependent overactivation of calpains occurs during reperfusion after pHi normalization makes them suitable pharmacological targets for the prevention of reperfusion injury. It has been reported that the administration of calpain inhibitors during the acute phase of reperfusion is effective in reducing infarct size.[75,178,179] However, some of the data that have been generated when the effects of prolonged calpain inhibition are investigated have been more controversial. Whereas in some studies, calpastatin overexpression in diabetic and hypertensive models attenuates the development of cardiac hypertrophy,[180,181] in other studies chronic inhibition results in cardiomyocyte degeneration and heart failure.[182] Further confirmation of the effectiveness of calpain inhibition in more clinically relevant experimental models and the development of more selective inhibitors are needed to confirm the therapeutic potential of calpain inhibition.

Inhibition of MPT opening with cyclosporin A may, under some conditions, reduce the release of mitochondrial Ca^{2+} and attenuate hypercontracture.[111] Prolongation of acidosis by postconditioning is supposed to inhibit MPT.[183] Also, protection induced by stimulation of the PKG signalling pathway has been interpreted as being mediated by the inhibition of MPT,[184] although a direct effect of this pathway on SR Ca^{2+} handling is also likely to contribute.[100,185] It is important to remark that the mechanisms by which inhibition of MPT may prevent sarcolemmal disruption in the first minutes of reperfusion, when the cytosolic Ca^{2+} concentration remains abnormally elevated, is not known.

6. Clinical studies

Even though L-type Ca^{2+} channels may play some role in the development of Ca^{2+} overload in ischaemic cardiomyocytes (see above), there is no evidence of a protective effect of Ca^{2+} channel blockers in the setting of acute myocardial infarction. In a small clinical study ($n = 36$), intracoronary nisoldipine given at the onset of reperfusion increased the ejection fraction.[186] In contrast, in the DATA trial ($n = 59$), no effects on ejection fraction or enzyme release were observed after i.v. administration of diltiazem.[187]

No NCX inhibitors have been approved for human use, and thus, there are no clinical trials on this target. However, calderet (MCC-135) is an intracellular Ca^{2+} handling modulator that is expected to inhibit, among other targets, the reverse-mode NCX and SR Ca^{2+} uptake. Calderet has been shown to reduce infarct size in the *in situ* dog heart when given i.v. at the end of ischaemia.[188] In the CASTEMI and EVOLVE randomized trials ($n = 387$), i.v. administration of calderet (MCC-135) did not modify infarct size or ejection fraction.[189,190]

The pharmacologic approach that has been more widely tested in patients to limit Ca^{2+} overload is NHE inhibition. The GUARDIAN study ($n = 11\,590$) did not show any benefit of cariporide on death or myocardial infarction in patients undergoing thrombolysis or coronary revascularization.[191] Similar lack of efficacy after eniporide administration at reperfusion was reported in the ESCAMI trial ($n = 1389$).[192] This lack of efficacy when given at the onset of reperfusion is in contrast with results obtained in trials in which NHE inhibitors were given before ischaemia, as in the EXPEDITION study,[193] and are fully consistent with preclinical studies showing that NHE inhibition protects against ischaemic injury but not against reperfusion injury, i.e. these agents need to be given before ischaemia to be effective.

Improved Ca^{2+} handling could be beneficial based on the positive results of the J-WIND-ANP trial,[194] in which patients with acute myocardial infarction receiving reperfusion therapy were given i.v. ANP or placebo. Patients treated with ANP showed a reduction in infarct size and an increase in ejection fraction. On the other hand, clinical evidence regarding the use of NO donors in the setting of acute myocardial infarction remains inconclusive: intracoronary sodium nitroprusside has been shown to reduce major adverse cardiac events,[195] whereas no benefit was observed with isosorbide mononitrate.[196]

Finally, pharmacologic inhibition of Ca^{2+}-induced MPT with cyclosporin A has been found able to prevent cell death during reperfusion in patients with acute myocardial infarction submitted to coronary angioplasty.[197] However, these promising results await confirmation in larger clinical trials.

In summary, there is a lack of clinically promising therapeutic strategies specifically addressing Ca^{2+}-mediated myocardial reperfusion injury, but in all strategies found to be protective in patients the beneficial effect can be explained at least in part by well-demonstrated effects on Ca^{2+}-mediated injury. A clear example of this is ischaemic postconditioning, a sequence of brief periods of ischaemia–reperfusion induced by repeated cycles of balloon-catheter inflation and deflation immediately applied after reopening of an occluded vessel. In experimental studies, ischaemic postconditioning has been shown to prolong acidosis and activate PKG signalling,[177] which result, through the mechanisms discussed above, in improved Ca^{2+} handling

and reduced Ca^{2+}-mediated calpain activation, hypercontracture, and permeability transition.[122]

7. Other potential modulators of Ca^{2+} handling during reperfusion

Ca^{2+}-mediated reperfusion injury could be modulated by age, sex, co-morbidities, and life style, and other clinically relevant conditions. The reported reduced tolerance to ischaemia of aged hearts[198] appears to be partially due to impaired Ca^{2+} handling. Aged hearts showed worsened Ca^{2+} overload and reduced functional recovery in isolated heart models.[199] The ability of SR to take up Ca^{2+} appears to decrease with advanced age, while the contribution of NCX appears to increase.[200] However, the effect of age could be different on different pathways of reperfusion injury. A recent report describes attenuated hypercontracture and necrosis and improved functional recovery after brief (30 min) global ischaemia in adult when compared with young guinea-pig hearts.[201]

There is ample evidence that sex may influence Ca^{2+} handling and tolerance to ischaemia–reperfusion injury. Testosterone increases Ca^{2+} transient amplitude and accelerates Ca^{2+} removal during relaxation,[202] and ovariectomy increases RyR Ca^{2+} release and NCX activity in rat hearts.[203] Part of the influence of sex on Ca^{2+} handling and cardioprotection could be mediated through enhanced PIK3-Akt-mediated PKG signalling in female hearts.[204] Of course, the protective effect of estrogens on reperfusion injury may be mediated to a large extent by primarily Ca^{2+}-independent mechanisms.[205]

Lifestyle (diet, exercise, stress, contamination) influences Ca^{2+} regulation in ways that can modify myocardial injury during ischaemia–reperfusion, and it may contribute to their effect on cardiovascular mortality. Animal studies and epidemiological evidence are consistent with the notion that exercise, when intense enough (approximately 75% of maximal oxygen consumption), activates a protective phenotype that limits infarct size secondary to subsequent transient coronary occlusion[206] and reduces spontaneous Ca^{2+} waves in post-infarction failing cardiomyocytes.[207] Exposure to carbon monoxide (CO) reduces tolerance to ischaemia in part by reducing SERCA and thus altering SR function,[208] in agreement with epidemiological evidence linking mortality secondary to myocardial infarction with exposure to urban CO.

8. Conclusions

Reperfusion injury has become an important part of the current scientific paradigm of tissue damage secondary to ischaemia in the heart, and this applies also to other organs. At the same time, the role of alterations in intracellular Ca^{2+} handling in its genesis has lost weight in favour of other mechanisms that can operate, mainly through MPT. Although the importance of these mechanisms is unquestionable, available data indicate that, in cardiomyocytes, altered Ca^{2+} handling may directly cause cell death through calpain-mediated proteolysis, hypercontracture, and, likely, MPT. These mechanisms appear to be critical in necrotic cell death occurring during the initial minutes of reperfusion, particularly after brief periods of ischaemia. Developing effective and safe treatments to prevent Ca^{2+}-mediated cell death in patients with acute myocardial infarction remains a challenge that needs to be met. The main difficulty in this task is the small-time window after reperfusion onset during which Ca^{2+}-mediated injury can be prevented. However, the widespread use of primary angioplasty in patients with acute myocardial infarction offers the opportunity to selectively apply treatments to the area at risk at the very onset of reperfusion.

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