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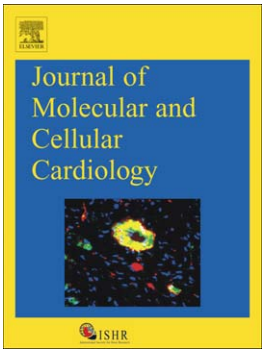

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Molecular identity of the mitochondrial permeability transition pore and its role in ischemia-reperfusion injury

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Running title: What is the mPTP, and what role does it play in ischemia-reperfusion?

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Abstract

The mitochondrial permeability transition is a key event in cell death. Intense research efforts have been focused on elucidating the molecular components of the mitochondrial permeability transition pore (mPTP) to improve the understanding and treatment of various pathologies, including neurodegenerative disorders, cancer and cardiac diseases. Several molecular factors have been proposed as core components of the mPTP; however, further investigation has indicated that these factors are among a wide range of regulators. Thus, the scientific community lacks a clear model of the mPTP. Here, we review the molecular factors involved in the regulation and formation of the mPTP. Furthermore, we propose that the mitochondrial ATP synthase, specifically its c subunit, is the central core component of the mPTP complex. Moreover, we discuss the involvement of the mPTP in ischemia and reperfusion as well as the results of clinical studies targeting the mPTP to ameliorate ischemia-reperfusion injury.

Keywords:

Mitochondrial permeability transition pore; ATP synthase; c subunit; cell death; apoptosis; ischemia-reperfusion; myocardial infarction.

Abbreviation list

ADP: adenosine diphosphate; ANT: adenine nucleotide transporter; ATP: adenine triphosphate; C1QBP: Complement component 1 Q subcomponent-binding protein; Ca^{2+}: calcium; CK: creatine kinase; CsA: cyclosporine A; CYCLE: CYCLosporinE A in reperfused acute myocardial infarction; ER: endoplasmic reticulum; ETC: electron transport chain; FADD: Fas-activated with death domain; FLIP: FLICE-inhibitory protein; GIK: glucose-insulin-potassium; GLP-1: glucagon-like peptide 1; GSK3-β: glycogen synthase kinase 3 beta; HF: heart failure; HK: hexokinase; Hot-DOG: ^{3}H 2-deoxyglucose; IHD: ischemic heart disease; IF-1: inhibitor protein F1; IMM: inner mitochondrial membrane; IMS: intermembrane space; IRI: ischemia-reperfusion injury; K^{+}: potassium; LV: left ventricular; Mg^{2+}: magnesium; MI: myocardial infarction; MITOCARE: prospective, multicenter, randomized, double-blind, placebo-controlled, phase IIa study; MPT: mitochondrial permeability transition; mPTP: mitochondrial permeability transition pore; mtCypD: mitochondrial cyclophilin

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D; MRI: magnetic resonance imaging; mTOR: mammalian target of rapamycin; MVO: microvascular obstruction; Na^+: sodium; NO: nitric oxide; OMM: outer mitochondrial membrane; OSCP: oligomycin sensitivity conferring protein; OXPHOS: oxidative phosphorylation; PCI: percutaneous coronary intervention; PEG: polyethylene glycol; P_i: inorganic phosphate; PiC: inorganic phosphate carrier; PM: plasma membrane; PPIF: peptidylprolyl isomerase f; PK11195: N-butan-2-yl-1-(2-chlorophenyl)-N-methylisoquinoline-3-carboxamide; PK: protein kinase; RISK: reperfusion injury survival kinase; RO5-4864: 4'-chlorodiazepam**;** ROS: reactive oxygen species; SAFE: survivor activating factor enhancement; SR: sarcoplasmic reticulum; STEMI: ST elevation myocardial infarction; TIMI: Thrombolysis in MI; TNFα: tumor necrosis factor alpha; TNFR1: TNF receptor 1; TRAIL: TNF-related apoptosis-inducing ligand; TRO40303: 3,5-Seco-4-nor-cholestan-5-one oxime-3-ol; TSPO: translocator protein; VDAC: voltage-dependent anion channel.

Highlights

- The c ring of mitochondrial ATP synthase is a critical component of the mPTP
- Mitochondria play a key role in necrosis and apoptosis in myocardial infarction
- mPTP is an important player in ischemia-reperfusion injury
- Ischemia-reperfusion injury induces dramatic increases in mitochondrial permeability
- mPTP represents an important therapeutic target to treat myocardial ischemia-reperfusion injury

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1. Ischemia-reperfusion injury (IRI): introduction and clinical background.

Ischemic heart disease (IHD) is the leading cause of death in Western countries. Each year, approximately 17 million people worldwide suffer from myocardial infarction (MI), and in 40% of cases, an ST segment elevation MI (STEMI) is presented [1]. Recent developments in myocardial reperfusion technique (e.g., primary percutaneous coronary intervention (PCI)) and in antithrombotic therapies permitted a significant improvement in the long-term outcome of STEMI patients [1]. Nevertheless, the mortality and disability associated with STEMI remain high [2] for several reasons, including a lack of therapy compliance and the under-use of specific cardiovascular drugs. Contemporaneously, the effectiveness of myocardial reperfusion remains a principal issue. It is estimated that approximately 50% of the final infarcted area is related to IRI [3], which consists of cardiomyocyte death following the restoration of blood flow in the related infarcted artery. IRI is strongly related to infarct size and to left ventricular (LV) remodeling. Both of these processes are known in daily clinical practice as strong and independent predictors of prognosis, heart failure (HF) and mortality [4].

Several clinical, cellular and molecular events occur during IRI (Figure 1). The most relevant clinical events are as follows: reperfusion-induced arrhythmia, myocardial stunning, microvascular obstruction (MVO), and myocardial necrosis secondary to reperfusion (Figure 1). The latter two entities are particularly well understood and are associated with increased infarct size and LV dysfunction severity. MVO is a phenomenon that occurs due to the following changes: capillary damage induced by vasodilatation, external compression caused by endothelial cell and cardiomyocyte swelling, micro-embolization of friable material released from the atherosclerotic plaque, and infiltration of inflammatory cells [5]. The myocardial necrosis that occurs secondary to reperfusion includes apoptosis and necrosis of cardiomyocytes and endothelial cells at a higher percentage than expected, resulting in a complete loss of the benefits of myocardial reperfusion via PCI [3]. In recent years, the complex mechanism that promotes the onset of IRI has been extensively studied but is currently only partially understood. This field of research is commonly referred to as “cardioprotection”, and various drugs and strategies have been initially evaluated using animal models and subsequently evaluated via clinical studies in humans in an attempt to reduce infarct size and thereby improve long-term prognosis. Recently, new advancements and discoveries in “cardioprotection” research have been reported. In particular, these advancements involve the role, function and structure of the mitochondrial permeability transition pore (mPTP)

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(Figure 2). Indeed, the mPTP (specifically, its opening) plays a key role in the development of myocardial necrosis that occurs secondary to reperfusion.

2. MPTP structure and the c subunit of mitochondrial adenosine triphosphate (ATP) synthase

2.1 Core components of the mPTP

It has been widely accepted that the permeability of the mitochondrial inner membrane is extremely low; thus, the discovery of a non-specific permeability transition with a threshold of 1.5 kDa suggested the existence of a pore (called the mPTP) that was responsible for this transition [6].

The initial clue for the existence of the mPTP came from the very early studies of Haworth and Hunter, which suggested that a hydrophilic channel was responsible for the permeability transition induced by polyethylene glycol (PEG) polymers of size up to 1.5 kDa [7]. This idea was confirmed by Crompton and Costi in 1988, who showed how, in its opened state, the mPTP channel should obtain a diameter of 2 - 2.6 nm [8]. Later electrophysiological studies performed by Zoratti's group identified the putative mPTP as the giant channel that, at that time, was known as the mitochondrial megachannel [9,10].

The initial evidence about the sensitivity of the mPTP to ADP and to the adenine nucleotide transporter (ANT) inhibitor atractyloside suggested a role for the ANT in the regulation of mPTP, and this finding was further supported by several other studies that involved identifying MPT sensitivity to other ANT ligands such as bongkrekic acid, palmitoyl-CoA and carboxy- atractyloside [11,12].

This idea was confirmed by a study of Halestrap and Davidson [13], who clearly displayed the correlation between ADP, ATP, Bongkrekic acid and carboxy-atractyloside with Ca^{2+}-induced MPT induction or in combination with cyclosporine A, an already well-known MPT inhibitor at that time [14–16]. Furthermore, in this initial study, Halestrap and Davidson proposed for the first time a model for the mPTP structure that involved the conformational state of the ANT and its interaction with the CsA target mitochondrial cyclophilin D (mtCypD) [13].

This model was supported by observations indicating that reconstituted ANT generates oligomers with properties analogous to the mPTP in artificial membranes [17,18].

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Shortly thereafter, electrophysiological studies proposed that two molecules of the voltage-dependent anion channel (VDAC) were components of the mPTP [9]. The involvement of the VDAC in the mPTP structure suggested that it might not be a common pore but rather a more complex and highly organized structure that included contact sites between the inner mitochondrial membrane (IMM, where the MPT actually occurs) and the outer mitochondrial membrane (OMM, where the VDAC is located).

This concept was demonstrated in 1996 by Brdiczka's group, who observed the existence of a protein complex that included the VDAC, ANT, hexokinase I (HK) and creatine kinase (CK, in its octameric form) and that displayed MPT activities when reconstituted in liposomal vesicles [19,20].

Due to its pharmacological properties, a protein of particular relevance for solving the molecular identity of the mPTP was mtCypD [21]. mtCypD can be inhibited by CsA, which has similar but opposite binding sensitivities to Ca^{2+} and ADP [22]. Additionally, mtCypD was shown to bind both the VDAC and the ANT [23]. The generation of transgenic mice lacking the peptidylprolyl isomerase f *(ppif)* gene confirmed that mtCypD is the protein element of mPTP that confers sensitivity to CsA [24,25]. Further, its cysteine 203 appears to have critical importance, especially regarding the sensitivity of mPTP to reactive oxygen species (ROS) [26]. Nonetheless, mtCypD is a mitochondrial matrix protein; thus, it is unable to generate a pore, and its depletion does not deny the existence of an MPT but rather dramatically increases the threshold for Ca^{2+} induction [27].

For a long time, the mPTP model proposed the VDAC and the ANT, which are located in the OMM and IMM, respectively, as the core components of the mPTP. These components have been proposed to be linked via a CK bridge [20]. mtCypD is also included in this complex as a regulatory element in the mitochondrial matrix [28].

However, two different studies based on knockout animal models challenged this model. The first study was performed using ANT1 and ANT2 double-knockout mice, and it demonstrated that the MPT occurs despite the loss of the ANT, even though these mice exhibit a loss of sensitivity to ANT inhibitors (bongkrekic acid or atractyloside) and a reduction in the Ca^{2+} threshold for mPTP opening [29]. The second study performed by Molkentin's group was based on a triple VDAC knockout model, which did not display any significant differences in either the Ca^{2+} threshold for mPTP induction or in cell death in response to various types of stimuli [30].

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These findings prompted a reconstruction of the structural mPTP model. Assuming that the ANT is not the pore-forming element on the IMM (it should be mentioned that two additional ANT isoforms have been identified after the ANT1/2 KO study), the VDAC-CK-HK-ANT complex should be interpreted only as a functional regulator of mPTP activity. In an early study, Brdiczka's group proposed that this complex may be fundamental for channeling adenine nucleotides [31] across the mitochondrial membrane, thus facilitating faster diffusion [19]. This hypothesis is supported by the loss of sensitivity of the MPT to ADP in ANT1/2 KO mice [29].

It has long been known that inorganic phosphate sensitizes the MPT pore, suggesting that the mPTP could possess a Pi-binding site. Inorganic phosphate is transported to the mitochondrial matrix by the mitochondrial phosphate inorganic carrier (PiC). In support of this concept, Leung and colleagues determined that the PiC interacts with mtCypD and the ANT [32]. Furthermore, this interaction is strengthened by MPT-inducing agents, whereas MPT-blocking compounds diminish this interaction. In the same year, based on a genetic screen, another group determined that PiC overexpression induces mitochondrial dysfunction and apoptosis [33]. These results, together with the earlier finding that a nonspecific pore is generated in liposomes by reconstituting the PiC [34], identified PiC as a strong candidate for the core-forming element of the mPTP.

This idea was well accepted until last year, when the same group performed PiC silencing experiments and found that knockdown of up to 70% of this carrier does not lead to any significant alteration in the Ca^{2+} threshold for the MPT, suggesting that either a small amount of PiC is required in the mPTP structure or that PiC is not a component of this structure [35].

The same concept was recently confirmed by the Baines and Molkentin groups, who generated cardiac-specific mouse strains whereby mitochondrial PiC levels could be genetically manipulated by overexpressing or knocking out/knocking down the *slc25a3* gene [36,37]. Both studies indicated that mPTP activity is not lost during PiC silencing or knock out and showed differences in its possible role as regulator. In fact, whereas the first study found no alterations in the mitochondrial Ca^{2+} retention capacity during either overexpression or silencing, the second showed that *slc25a3* deletion results in increased Ca^{2+} retention capacity (less-sensitive mPTP) and protects cells from stimuli able to induce MPT. Furthermore, the KO mouse was protected by reperfusion injury compared to the control, confirming its role as a regulator of the mPTP (a critical player during heart reperfusion injury, see below). Identifying what variables could generate the differences

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observed by these studies could be difficult, but overall, they confirm that PiC should not be considered a structural component, but rather a minor regulator, of the mPTP.

2.2 mPTP regulatory elements

Although the minimal structure required for mPTP activity is uncertain, a plethora of mPTP regulators have been identified.

One of the first regulators to be discovered was the mitochondrial translocator protein (TSPO), an 18-kDa protein that is localized in the OMM; this protein, together with the VDAC and the ANT, was initially identified as a component of the peripheral benzodiazepine receptor [38]. The interactions of TSPO with the VDAC and the ANT indicate that it is a possible component of the mPTP, and this hypothesis was supported by Sileikyte and co-workers in 2011 [39]. The effects of TSPO on mPTP activity remain controversial because opposite outcomes have been observed for some of its ligands (as RO5-4864 and PK11195) in different studies [40–44]. Of note, TSPO ligands have been shown to display pro-apoptotic effects, even during TSPO silencing, which likely results from the expression of other benzodiazepine receptors [43]. Recently, Sileikyte and co-workers updated their findings in mouse liver through the use of a conditional TSPO KO. In this study, they indicated that TSPO is not a requirement for the OMM to regulate the MPT, but can exert only minor regulatory effects [45].

The crucial importance of the MPT in cell death is indicated by the participation of Bcl-2 family members in the formation of the mPTP. Bax and Bak are well known pro-apoptotic members of the Bcl-2 family that translocate to the OMM to induce mitochondrial depolarization and cytochrome C release, even in isolated mitochondria [46–48], which implicates the involvement of Bax and Bak in the formation of the mPTP. In 1998, two independent groups demonstrated that both proteins interact with the mPTP to induce the MPT and release of cytochrome C, and these studies indicated that this process requires cooperation with the ANT [49,50]. These data were confirmed by Molkentin's group, who used genetic background knockout models for Bax and Bak [51]. Molkentin's group proposed that regulation of the MPT by Bak and Bax is dependent on their ability to permeate the OMM, represents a minimal requirement for the induction of mitochondrial swelling and occurs independent of the ANT [51]. In the future, this model should be validated using an ANT knockdown model. Additionally, the removal of Bax and Bak leads to impaired mitochondrial Ca^{2+} uptake [52], indicating that Bax and Bak can effectively cause

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impaired OMM permeability (assuming that this permeability affects Ca^{2+} transport across the OMM). During their stimulation, Bax and Bak can also increase the amount of free Ca^{2+} in the mitochondrial matrix (by promoting Ca^{2+} flux into the mitochondrial matrix) to trigger the MPT. Furthermore, Bad, which is a pro-apoptotic member of the Bcl-2 family, has been shown to induce the MPT in isolated mitochondria in a Bax- and Bak-independent manner [53]. In addition to these findings, anti-apoptotic members of the Bcl-2 family have been shown to modify MPT activity. For example, Bcl-2 and Bcl-XL have been shown to interact with the ANT and the VDAC, respectively [54,55].

One mPTP regulator that has attracted particular interest is glycogen synthase kinase 3 beta (GSK3-β) [56]. This protein contributes to many cellular processes, such as transcription, metabolism, cell division, adhesion and apoptosis. In 2004, it was proposed that GSK3-β functions as a convergence point for the inhibition of the MPT via different survival signaling pathways, including protein kinase A (PKA), protein kinase B (PKB), protein kinase C (PKC) and mammalian target of rapamycin (mTOR) [57]. This concept was supported by additional studies reporting that GSK3-β is a therapeutic target for cardioprotection [58–60]. The complete mechanism by which GSK3-β is involved in mPTP function has yet to be elucidated, and it is especially unclear if its kinase activity is required. Nonetheless, it has been shown that GSK3-β inhibitors impair adenine nucleotide transport across the matrix, which is related to a reduction in VDAC2 phosphorylation [61].

mPTP regulation via pro-survival kinase signaling has also been attributed to PKCε. This particular isoform has been associated with cardioprotection based on studies using transgenic mice [62]. Interestingly, the same group has shown that PKCε interacts with the VDAC1-HKII-ANT complex, resulting in VDAC1 phosphorylation and inhibition of mPTP activity. Additionally, PKCε has been reported to be able to reduce the Ca^{2+} content in the sarcoplasmic reticulum and decrease the risk of mPTP opening during reperfusion, thus providing a novel mechanism for preconditioning-mediated cardioprotection [63].

Several other proteins, such as PKG, p53, and Complement component 1 Q subcomponent-binding protein **(**C1QBP), have been proposed in at least one study to directly modulate mPTP activity [64–66]. Further elucidation of their role in the MPT is required.

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2.3 The critical role of the ATP synthase c-subunit in mPTP function

ATP synthase displays a series of characteristics upstream of its regulation that resemble those of the mPTP. First, the hydrolytic activity of ATP synthase is strongly inhibited by the concurrent binding of two mPTP inhibitors, namely ADP and Mg^{2+}, to its catalytic site, the so-called Mg-ADP block [67], but the mPTP inducer Pi has been proposed to abolish this block. Second, two different cysteine residues (C294 in the alpha subunit and C103 in the gamma subunit) may be linked by a disulfide bridge during oxidative stress, thereby impeding ATP synthase activity [68]. Furthermore, the ATP synthase complex forms a supercomplex with the ANT and PiC, both of which have been proposed as components of the mPTP, and this complex is referred to as the ATP synthasome [69,70]. In 2009, mtCypD, another regulatory component of the mPTP, was shown to interact with the peripheral stalk of ATP synthase, particularly the oligomycin sensitivity conferring protein (OSCP) and d subunits. These interactions result in reduced catalytic activity (both hydrolase and synthase) that can be restored by displacing mtCypD with CsA [71]. Finally, anti-apoptotic Bcl-XL, a known MPT inhibitor, interacts with ATP synthase and promotes its synthetase activity [72].

Physiological studies also suggest a correlation between ATP synthase and the mPTP. The c-ring-selective inhibitor, oligomycin, prevents both tumor necrosis factor alpha- (TNFα) and Bax-induced MPT and cell death [49,73].

Recently, we identified the c subunit of the mitochondrial ATPase as a fundamental regulator of mPTP activity [74,75]. Of all of the subunits that compose the F_O complex (see above), the a, b and c subunits are sufficient to facilitate the translocation of protons across lipid bilayers, and these subunits are highly evolutionarily conserved, as previously mentioned [76,77].

It has recently been shown that Rho0 cells, which lack mitochondrial DNA, are equipped with a functional mPTP [78]. This finding excludes a role for the a subunit of the mitochondrial ATP synthase in the mPTP. Furthermore, conductive properties have only been ascribed to the c subunit [79], and a peptide displaying a consistent degree of similarity to the c subunit has been proposed as a putative regulator of the mPTP [41,80], thus indicating that the c subunit is the best candidate for a pore component.

Furthermore, we found that silencing c subunit expression completely blocks MPT induction by Ca^{2+} and oxidants, whereas c subunit overexpression dramatically enhances MPT induction. Silencing the c subunit does not affect ATP synthesis, suggesting that MPT inhibition is not due to

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the accumulation of ADP in the mitochondrial matrix. Furthermore, silencing α subunit expression does not lead to any significant alteration in MPT activity, suggesting that the c subunit of the mitochondrial ATP synthase is a central component of the mPTP. In support of our results, it has been recently reported that the isolated c subunit induces the MPT in isolated mitochondria and forms ion channels in artificial bilayer membranes. Furthermore, this activity is stimulated by Ca^{2+}, inhibited by CsA and dependent on the phosphorylation state of the c subunit [81].

Nonetheless, it has yet to be validated that c-rings exist on the outside of ATP synthase, leaving the c-ring unoccupied by the central stalk and thus available to generate currents in vivo.

Interestingly, a few months after our publication, Bernardi's group confirmed the regulatory role of ATP synthase in mPTP function and suggested that only ATP synthase dimers exert mPTP-like activity when inserted into a lipid bilayer [82]. However, this concept contrasted findings published by the same group that showed that MPT characteristics are also detected in Rho0 cells depleted of mitochondrial DNA [78]. Indeed, Wittig et al. demonstrated that Rho0 cells contain unstable oligomeric (and dimeric) structures of ATP synthase at extremely reduced levels [83].

Dimerization of ATP synthase is favored and stabilized by the inhibitor protein F1 (IF-1), and this event is associated with increased ATP production and reduced susceptibility to cell death during ischemia [84]. Reduced dimerization is detected in aging cells, thus favoring cell death. Furthermore, dimer dissociation is reduced by mtCypD, and CsA impedes the transition from dimers to monomers, thereby suggesting that the dimer itself is likely not the mPTP but rather that the transition from dimers to monomers favors mPTP formation [85]. Giorgio et al. showed that ATP synthase dimers extracted from a native gel prior to insertion into a lipid bilayer could produce some monomers, most likely due to technical manipulation during complex extraction. Therefore, it is possible that an unstable monomer generated during this procedure could rearrange under appropriate conditions to generate the mPTP based on the c rings of ATP synthase. This notion is supported by a recent publication demonstrating that the c-ring can generate a non-specific current ascribable to the mPTP and as isolated F1/FO ATP synthase monomers reconstituted on vesicles generate mPTP-like currents when bound to mtCypD and exposed to Ca^{2+} [86]. Moreover, it has been suggested as the Ca^{2+}-induced mitochondrial swelling can at least partially detach the F1 subunit from the FO subunit, and this detachment can be reversed by CsA [86].

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3. Necrosis and apoptosis, mitochondria and the mPTP

Mitochondria are important dynamic organelles that function as the gate-keepers of life and death. In cardiac myocytes, mitochondria occupy up to 30% of the total volume, as these cells have a large energy requirement in the form of ATP via oxidative phosphorylation (OXPHOS) to maintain their functional integrity [87].

Mitochondria, the powerhouses of the cell, are sensitive to alterations in the cellular environment and can quickly switch from a sustainer of cell survival to a promoter of cell death via the necrotic or apoptotic pathways [88]. Therefore, it is not surprising that mitochondrial dysfunction is associated with the loss of myocytes and the subsequent development of HF.

Necrosis and apoptosis differentially contribute to MI. Both processes are regulated by many of the same biochemical intermediates, including alterations in the levels of high-energy phosphates, intracellular Ca^{2+}, and ROS.

3.1 Necrosis

Necrosis is generally considered to be initiated by non-cellular mechanisms, such as ischemia, trauma, and thrombosis, which ultimately lead to irreversible cell death (Figure 3). This cell death is characterized by cell swelling, depletion of high-energy stores and disruption of the cellular membrane, which involves alterations in fluid levels, alterations in electrolyte levels, loss of potassium ions (K^+), loss of Mg^{2+} ions, and the intracellular accumulation of water, sodium ions (Na^+), chloride ions (Cl^-), protons (H^+), and Ca^{2+} ions [89,90]. During ischemia, anaerobic metabolism is predominant due to energy failure, thus producing a decrease in intracellular pH. To buffer this accumulation of hydrogen ions, the Na^+/H^+ exchanger excretes excess hydrogen ions, which produces a large influx of Na^+ [91]. Indeed, ischemia depletes cellular ATP, which inactivates ATPases (e.g., Na^+/K^+ ATPase), reduces active Ca^{2+} efflux, and limits the reuptake of Ca^{2+} by the sarcoplasmic reticulum (SR), thereby producing intracellular Ca^{2+} overload. In the heart, these cellular changes are accompanied by the activation of intracellular proteases (e.g., calpains) that damage myofibrils and induce hypercontracture and contracture band necrosis. This type of cell death is also referred to as passive necrosis.

In the 1980s, Crompton et al. were the first to propose a pivotal role of MPT in cardiac IRI [92]; as a working hypothesis, they proposed that the changes in Ca^{2+}, P_i and adenine nucleotide levels

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during ischemia trigger mPTP opening [14,92,93]. Griffiths and Halestrap subsequently demonstrated that MPT occurs upon reperfusion of the ischemic heart. In 1995, using the mitochondrial 'Hot DOG' – entrapment technique, they showed that some mitochondria can undergo mPTP opening and closure in the ischemic-reperfused heart [94]. Their data confirm that pore opening occurs during reperfusion of the heart after ischemia, but not in the ischemic priming period. Their experimental procedures tell us that the extent of DOG uptake increases until the period of ischemia that precedes reperfusion increases to an empirical maximum of 30-40 min [94].

Opening of the mPTP facilitates the free passage of protons across the IMM, leading to a dissipation of the mitochondrial membrane potential and pH gradient, which comprise the proton motive force. Not only does this process prevent ATP generation, but reversal of the ATPase also occurs, thus causing the breakdown of cytosolic ATP that is generated via glycolysis. Energy metabolism is further impaired, thereby resulting in a continuous cycle of increasing Ca^{2+} deregulation and mPTP opening. These changes activate phospholipases, nucleases and proteases.

The importance of the mPTP in the necrotic death of cardiomyocytes under such conditions was initially detected in experiments using mPTP inhibitors, such as CsA [14,90]. Recently, further evidence for a critical role of mPTP opening in necrotic cell death has been provided by the use of mice in which the target of CsA, mtCypD, was knocked out [89,91]. These animals exhibit substantial protection from IRI-induced damage (infarct size) to the heart. In addition, using these mice, it has been shown that cardiac failure associated with chronic Ca^{2+} overload involves the mPTP-dependent death of cardiomyocytes [95]. At last, in 2014, a study of sixty-one patients that was directed by Ovize showed that cyclosporine administration at the time of reperfusion protects against reperfusion injury in patients undergoing aortic valve surgery by reducing the levels of cardiac troponin I in the cyclosporine group compared with the control group [96]. Most of these concepts have been widely reviewed [97,98].

Today, MI, bypass surgery and organ transplantation provide dramatic examples of this mechanism of cardiac failure. This step in cell death involves the mPTP and a complex network of cellular signals. Because the severity of the insult in most infarction cases in the heart is heterogeneous, there is often no clear boundary between apoptosis and necrosis. However, if the stress experienced by the cell is a severe insult, the extent of mPTP opening is catastrophic, and

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necrotic cell death is inevitable, as occurs in the core of vessel obstruction. In this region, most mitochondria undergo massive matrix swelling and OMM rupture.

3.2 Apoptosis

In addition, reperfusion can lead to an enhancement in apoptosis [95], which is an evolutionarily conserved mode of cell death that can be initiated via two different pathways in mammals: the death receptor pathway (extrinsic apoptotic pathway) and the mitochondrial pathway (intrinsic apoptotic pathway). Furthermore, the apoptosis pathway that is activated depends on the nature of the death signal (Figure 3). Apoptosis, similarly to necrosis, can be induced by mPTP opening. For apoptosis, the stress is often a milder insult than that for necrosis, which could explain the apoptotic ring around the necrotic core of a coronary infarct [99]. mPTP opening might be transient or maintained in some mitochondria undergoing matrix swelling, where all small-molecular-mass solutes equilibrate across the IMM, and proteins remain at a high concentration in the matrix and exert colloidal osmotic pressure that unfolds the IMM cristae and induces OMM rupture [100,101].

3.2.1 Extrinsic apoptotic pathway

Mitochondrial membrane permeabilization does not play a crucial role in the extrinsic pathway. Instead, it is most likely activated in response to inflammation that is required for healing and scar formation in the infarct. Plasma membrane receptors are activated by pro-inflammatory ligands, including Fas, TNF-α and TNF-related apoptosis-inducing ligand (TRAIL).

Fas and Fas ligands are expressed in the heart and enhanced expression of Fas is associated with increased apoptosis in experimental models of MI [102,103]. Simulated IRI in a cell culture model increases the sensitivity of myocytes to Fas-mediated death. Therefore, it has been suggested that IRI might down-regulate inhibitors of the Fas pathway, such as cellular FLICE-inhibitory protein (cFLIP). cFLIP is highly expressed in the heart under normal physiological conditions but is degraded after IRI. Thus, the loss of cFLIP expression may be important for enhancing the sensitivity of cardiomyocytes to apoptosis after IRI. These results suggest that the Fas-mediated cell death pathway exists in cardiomyocytes but that under normal conditions, this pathway is

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down-regulated by inhibitors. However, after stress, such as ischemia, cFLIP becomes inactivated, thus rendering the cells susceptible to death via the Fas pathway.

Recent studies have revealed that TNF plays a role in the progression of myocardial disease. Increased TNF-α and TNF receptor 1 (TNFR1) expression levels are associated with HF [104]. As TNF-α induces apoptosis in cardiomyocytes [105], it is thought that at least part of its pathogenic effect in the heart is due to its induction of cell death.

In contrast, there is also evidence supporting a prosurvival role of TNF in the heart, including the involvement of TNF in the regulation of adaptive responses to biomechanical stress. Examples of these adaptive responses include the induction of cellular hypertrophy in response to pressure overload and the modulation of contractile function following ischemia.

The role of inflammation in MI as a target for cardioprotection has not been completely addressed. A small number of studies have investigated the effects of reducing the inflammatory response to myocardial reperfusion injury. Experimental animal studies have reported significant reductions in MI size with several interventions administered at the time of myocardial reperfusion, such as the inhibition of neutrophil aggregation and attenuation of leukocyte infiltration into the infarcted myocardium [106,107]. On the other hand, more recently, clinical studies targeting the inflammatory components of MI have failed to show a significant improvement in reperfused–STEMI patients [108]. Further studies on the role of inflammation in MI are required.

3.2.2 Intrinsic apoptotic pathway

Ca^{2+} is a critical sensitizing signal for the pro-apoptotic transition of mitochondria that plays a key role in the regulation of cell death [109]. Mitochondrial Ca^{2+} overload is a pro-apoptotic inducer of mitochondrial swelling, and OMM perturbation or OMM rupture leads to mitochondrial apoptotic factor (cytochrome c, Smac/DIABLO, AIF and Omi/HtrA2) release into the cytosol [110,111]. Cytochrome c-mediated apoptosis is important in cardiomyocytes. Serum and glucose deprivation induce cytochrome c release in vitro, thereby resulting in the activation of caspase-9, caspase-3 and apoptosis [112]. As serum and glucose deprivation are components of ischemia in vivo, these results indicate that this pathway may be involved in heart disease-related cell death.

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Most reperfusion-induced apoptotic death of cardiomyocytes occurs during the initial minutes of reperfusion due to increased ROS production, intracellular Ca^{2+} overload and mPTP opening [95]. The role of apoptosis in reperfusion injury has recently been addressed using rat and rabbit animal models in which reperfusion accelerates the occurrence of apoptosis in cardiomyocytes [95,113]. In the infarcted region of the ventricular wall, myocytes containing DNA strand breaks are detected 2 hours after coronary artery occlusion, and approximately 2.7 million myocytes are apoptotic at this time point. Moreover, 6.6 million cells are apoptotic at 4.5 hours, indicating that there is a 2.4-fold increase in the absolute number of apoptotic myocytes in the left ventricular free wall from 2 to 4.5 hours after coronary artery occlusion. The magnitude of apoptosis progressively decreases at later time intervals. Necrosis of myocytes also appears 2 hours after coronary artery occlusion and continuously increases until one day following coronary artery ligation. These findings demonstrate that myocyte apoptosis and necrosis are independent variables contributing to infarct size, although apoptosis accounts for 86% of the total loss of myocytes, and necrosis accounts for only 14% of the total loss [113].

In contrast, findings from other laboratories that support these experimental data indicate that MI results from a significant increase in necrosis rather than apoptosis, where pro-apoptotic factors are evident only early during ischemia but do not significantly contribute to infarct size [114,115]. Others have found that apoptosis and necrosis occurred simultaneously in all instances in hearts from cases of fatal MI [116]. One likely hypothesis that could explain the coexistence of apoptosis and necrosis after IRI is that damage produced by ischemia is capable of initiating apoptosis, but if ischemia is prolonged, necrosis ensues (as discussed later).

3.3 Role of ROS and Ca^{2+} in IRI-induced damage

A wide range of mitochondrial ROS-induced damage has been described, including protein carbonylation, lipid peroxidation and mitochondrial DNA damage [117]. These modifications are important factors in the progression of myocardial IRI-induced damage. The re-introduction of abundant oxygen at the onset of reperfusion evokes a burst of toxic oxygen derivatives within the first few minutes of reperfusion. Moreover, oxidative stress also reduces the bioavailability of nitric oxide (NO, a vasodilator) during reperfusion [118].

Cytosolic Ca^{2+} accumulation plays major roles in the initiation of programmed cell death during acute MI. A prolonged increase in cytosolic Ca^{2+} induces mitochondrial Ca^{2+} overload, which leads

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to mPTP opening and the activation of Ca^{2+}-dependent proteases [119]. Increased cytosolic Ca^{2+} plays a pivotal role in activating the serine threonine Ca^{2+}/calmodulin-regulated phosphatase, calcineurin. This phosphatase is a critical transducer of Ca^{2+} signals in most cell types, particularly in the heart, due to its specific responsiveness to sustained, low-frequency Ca^{2+} signals [120].

Biochemical events leading to mPTP opening during ischemia and reperfusion

The effects of ROS and Ca^{2+} on MPT have been widely reported as key players during ischemia and reperfusion damage [97,121] (Figure 3). During ischemia (the MPT-priming phase), the accumulation of factors, including Ca^{2+}, long-chain fatty acids and ROS, progressively increases the susceptibility to MPT, thus increasing the likelihood that MPT will occur upon reperfusion (the MPT-activating phase) [122].

Indeed, the conditions that occur during ischemia and reperfusion are identical to those that induce mPTP opening. During ischemia, increased glycolysis causes the accumulation of lactic acid and the reduction of pH. To restore the pH, the Na^+/H^+ antiporter is activated, but it acts inefficiently because Na^+ cannot be pumped out of the cell, as the Na^+/K^+ ATPase is inhibited by the absence of intracellular ATP. Consequently, the cytosolic Ca^{2+} concentration increases because the activity of the Na^+/Ca^{2+} antiporter is reduced or reversed. In addition, during ischemia, there is a decrease in the adenine nucleotide concentration, which is associated with an increased phosphate concentration, thereby sensitizing mPTP opening in response to Ca^{2+}; however, low pH inhibits mPTP opening. If the period of ischemia is prolonged, the heart becomes irreversibly damaged due to the activity of degradative enzymes, such as phospholipases and proteases, which also compromise mitochondrial function [123].

Upon reperfusion, the mitochondria recover their ability to respire and rescue the sustained mitochondrial membrane potential that is required for ATP synthesis. However, the mitochondrial membrane potential is the driving force for mitochondrial Ca^{2+} uptake, thus leading to Ca^{2+} overload. In addition, rapid and extensive production of ROS occurs when the inhibited respiratory chain is re-exposed to oxygen. Thus, the following resulting conditions are nearly optimal for mPTP opening: high Ca^{2+} levels within the mitochondrial matrix, increased levels of phosphate and oxidative stress, depletion of adenine nucleotide concentration, and rapid return of the pH to a physiological value [124,125].

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After ischemia and reperfusion, the fate of the cell is determined by the severity of the damage as follows: if the damage is minimal, the cell may recover; if the damage is moderate, the cell may undergo apoptosis; and if the damage is severe, the cell may die from necrosis due to inadequate energy production. Thus, mitochondria serve as an arbiter of cell fate in response to stress [119].

4. Clinical studies examining pharmacological agents to reduce IRI.

Considering the pivotal role of the mPTP in IRI during STEMI, many studies have focused their attention on pharmacological agents that modulate mPTP opening. Currently, a limited number of these agents act directly on mPTP and/or its components. Contrarily, the majority of these agents are able to influence biological parameters (e.g., ROS, pH and PI signaling pathways) that indirectly modulate the final stage of mPTP opening. Finally, several strategies of ischemic pre- and post-conditioning have been developed and studied to reduce IRI during STEMI [126]. Nevertheless, details of these studies are beyond the aim of this review. Hence, this review will only focus on the pharmacological approaches to cardioprotection in humans (Table 1).

Agents directly targeting mPTP

One of the most promising results in cardioprotection has been reported by Piot et al. using CsA [127]. Since the 1990s, it has been known that CsA inhibits mPTP opening by binding to mtCypD, a mitochondrial isomerase that binds to subunits b, d and O in the lateral stalk of the F1-F_o ATPase. Studying 58 patients, Piot el al. examined the effect of administration of an intravenous bolus of 2.5 mg/kg CsA to patients experiencing STEMI immediately before undergoing PCI by measuring the release of myocardial-specific enzymes and performing magnetic resonance imaging (MRI) on the infarcted heart within the fifth day after reperfusion. The results confirmed the cardioprotective effect of CsA and showed a significantly reduced overall infarcted area in the group treated with CsA compared with the control group [127].

The CYCLosporinE A in reperfused acute myocardial infarction (CYCLE) phase III clinical trial is currently underway and is designed to address the clinical effectiveness of CsA for STEMI during reperfusion therapy [128]. The role of CsA in cardioprotection has also been tested in cardiac surgery and after coronary artery bypass graft and after aortic valve surgery. In the first study, Hunseloy et al. demonstrated that a single intravenous bolus of CsA (2.5 mg/kg) administered

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prior to CABG surgery reduced the extent of perioperative myocardial injury, with a reduced postoperative cardiac troponin T rise by 0.03 ng/ml for every 10 minutes, when compared with the control (p=0.049) [129]. Additionally, in the setting of aortic valve surgery, the administration of CsA demonstrated a beneficial effect in reducing RI that was expressed as a 35% reduction in the area under the curve of cardiac troponin I compared with the control group (p=0.03) [96]. 3,5-Seco-4-nor-cholestan-5-one oxime-3-ol (TRO40303) is an mPTP modulator that binds to the mitochondrial translocator protein at its cholesterol site, which results in reduced release of apoptosis-inducing factors into the cytosol after ischemia and reperfusion [130]. This new drug is under evaluation in the MITOCARE trial to test whether its injection reduces infarct size, as measured via both cardiac biomarker release and MRI within the fifth day after primary PCI [131].

Agents indirectly targeting the mPTP

Many other substances that indirectly target the mPTP have been tested. Among the most interesting drugs that have been studied are exenatide, atrial natriuretic peptide, and glucose-insulin-potassium (GIK). Exenatide is an analog of glucagon-like peptide 1 (GLP-1), and a post-hoc study has demonstrated that this substance reduces the final infarct size by 30% in patients experiencing STEMI and Thrombolysis in MI (TIMI) flow grades of 0 or 1 based on angiogram [132]. However, this benefit was limited to patients with a rapid symptom-onset-to-balloon time (≤132 minutes). The cardioprotective effect of exenatide is unclear, but it has been recently proposed that GLP-1 also acts on the mPTP. The beneficial effect of atrial natriuretic peptide has been evaluated in a small, randomized trial [132]. This study enrolled patients experiencing acute MI who received PCI, and the atrial natriuretic peptide was administered as an adjunctive treatment (compared to placebo). The authors showed that the patients receiving atrial natriuretic peptide exhibited a 14.7% reduction in the infarct size (95% CI 3.0-24.9%) and a significant increase in the LV ejection fraction after 6-12 months. The effect of atrial natriuretic peptide on mPTP is most likely due to inactivation of GSK3-β [133]. Despite promising preliminary results (generally using animal models), randomized studies using other pharmacological agents have failed to demonstrate a clear benefit in reducing IRI or mortality (Table 1). Yellon et al. described the cardioprotective role of reperfusion injury survival kinase (RISK) and survivor-activating factor enhancement (SAFE), which are two pro-survival kinase pathways that converge on the mitochondria to reduce mPTP opening [134]. Accordingly, some authors have speculated that a GIK solution exerts a cardioprotective effect by modulating pro-survival kinase pathways via the

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GIK receptor, which is a G protein-coupled receptor [135]. Nevertheless, no clinical benefit has been observed in a confirmatory randomized clinical trial (Table 1) [136]. Finally, a new substance, namely Bendavia, is under evaluation. Bendavia is a peptide that interacts with cardiolipin in the IMM to reduce ROS production and maintain the efficiency of the electron transport chain during reperfusion [137]. The EMBRACE trial is ongoing to test the potential clinical application and effectiveness of Bendavia. The principal aim of the EMBRACE trial is to demonstrate that Bendavia injection will reduce infarct size, as assessed by analyzing cardiac biomarker release and MRI [137].

Overall, the current available data regarding pharmacological agents acting directly or indirectly on the mPTP and IRI are limited. Few trials have demonstrated a net clinical benefit but have been limited by a small sample size, the use of surrogate endpoints and extensive exclusion criteria.

As mentioned above, to evaluate the reduction in infarct size, all trials measured the biomarker levels, and the ejection fraction was determined based on echocardiography and magnetic resonance imaging. In the majority of cases, all patients underwent two MRI scans as follows: the first scan was performed within a week after primary PCI, and the second scan was performed at a follow-up visit. The measured parameters include the area at risk based on T2-weighted images, the final infarct size based on late-enhancement MRI sequences and the myocardial salvage index [(area at risk minus infarcted size)/area at risk] [138,139].

5. Conclusions.

IRI induces dramatic increases in mitochondrial permeability, thereby initiating a chain of events that leads to both apoptosis and necrosis of cardiomyocytes. Thus, the mPTP represents a therapeutic target to reduce cardiomyocyte mortality and treat myocardial IRI. Unfortunately, antagonizing the mPTP in the clinical setting has been hampered by the lack of a precise understanding of its molecular architecture.

Here, we propose a model in which ATP synthase is the central element of the mPTP as follows: (i) ATP synthase shares several activators and inhibitors; (ii) ATP synthase interacts with various regulators of the mPTP (including the ANT, PiC and mtCypD); and (iii) the c ring of ATP synthase (the lone subunit confirmed to display gating capacity) plays a critical role in mPTP activity. Further studies are required to achieve a complete understanding of the structure and activity of the mPTP. Finally, the recent discovery of several mPTP components provides novel targets for

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cardioprotection. Currently, the overall molecular identity of the mPTP remains unknown, but this information may facilitate the development of more specific and potent mPTP inhibitors.

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Table 1: Pharmacological approaches for cardioprotection in humans.

Study	*Ref.*	*Pts.*	*Therapeutic substance*	*Therapeutic protocol*	*Outcome*
Agents directly targeting mPTP					
Piot et al.	[127]	58	***Cyclosporin A***: Inhibitor of cyclophilin D, directly blocks mPTP opening.	Cyclosporin A (2.5 mg/kg iv) 10 min prior to primary PCI.	44% reduction of MI size (72-h AUC total CK), 20% reduction of MI size (MRI in a subset of 27 patients), 28% reduction of MI size and smaller LVESV on MRI at 6 months.
Hausenloy et al.	[129]	78	***Cyclosporin A***	Cyclosporin A (2.5 mg/kg iv) after induction of anesthesia but prior to sternotomy.	Reduction in perioperative myocardial injury with CsA (p=0.049) with the postoperative cardiac troponin T rise reduced by 0.03 ng/ml for every 10 min. No differences in mean peak cardiac troponin T between control and CsA treatment.
Chiari et al.	[96]	61	***Cyclosporin A***	Cyclosporin A (2.5 mg/kg iv) less than 10 min before aortic clamping.	35% reduction in AUC for cardiac troponin I in the CsA group compared with the control.
Latini et al.	[128]	444	***Cyclosporin A***	Cyclosporine A (2.5 mg/kg) 5 min before PCI.	Ongoing trial to investigate the improvement of myocardial reperfusion, measured with ST segment myocardial resolution ≥70% one hour after PCI.
Mitocare group	[131]	180	***TRO40303***: Binds mitochondrial translocator protein at the cholesterol site, modulates mPTP.	TRO40303 (35 ml/min iv) injection of TRO40303 15 min before balloon inflation and stenting.	Ongoing trials to investigate reduction in MI size (total CK and troponin AUC and myocardial salvage index on MRI).
Agents indirectly targeting mPTP					
Lønborg et al.	[132]	107	***Exenatide***: Analog of GLP-1.	Exenatide (25 mg in 250 ml saline, iv) 15 min prior to primary PCI and continued for 6 h.	23% reduction in MI size by AAR at 90 days by MRI (from 0.30 to 0.39), Increased myocardial salvage index (from 0.62 to 0.71), Reduced MI size in patients presenting with ischemic times <132 min (8% vs 11%).
Kitakaze et al.	[140]	569	***Atrial natriuretic peptide:*** Inactivation of GSK3-β, indirectly blocks mPTP.	Carperitide (0.025μg/kg/min iv) for 72 h after reperfusion.	14% reduction in MI size (total CK AUC), 2% increase in LVEF at 6-12 months.
Ross et al.	[108]	2118	***Adenosine***: Anti-inflammatory effect, reduction of oxygen-free radicals.	Adenosine (50-70 μg/kg/min iv) for 3 h after PCI.	No difference in death or HF at 6 months.
Kim et al.	[141]	171	***Atorvastatin***: Reduction of oxygen-free radicals.	Atorvastatin (80 mg oral) vs atorvastatin (10 mg oral) prior to primary PCI	No difference in death, MI size, revascularization, MI recurrence.
Selke et al.	[136]	357	***Glucose insulin potassium*** (GIK): Prevention of oxygen-free radical production.	GIK (iv) begun in the ambulance for suspected STEMI.	No difference in progression to MI, reduction of composite endpoint of cardiac arrest or in-hospital mortality (6.1% vs 14.1%).
Chakrabarti et al.	[137]	300	***Bendavia***: Interaction with cardiolipin, reduces ROS production.	Bendavia (0.05 mg/kg iv) between 15-60 min before PCI and continued for 1 h after revascularization.	Ongoing trials to investigate the reduction of MI size (total CK AUC and late-enhancement on MRI).

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Ref: reference. Pts: number of patients. mPTP: mitochondrial permeability transition pore. PCI: percutaneous coronary intervention. MI: myocardial infarction. CK: creatine kinase. AUC: area under the curve. MRI: magnetic resonance imaging. h: hours. AAR: area at risk. min: minutes. LVEF: left ventricle ejection fraction. HF: heart failure.

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Figure 1. Ischemia-reperfusion injury during acute myocardial infarction.

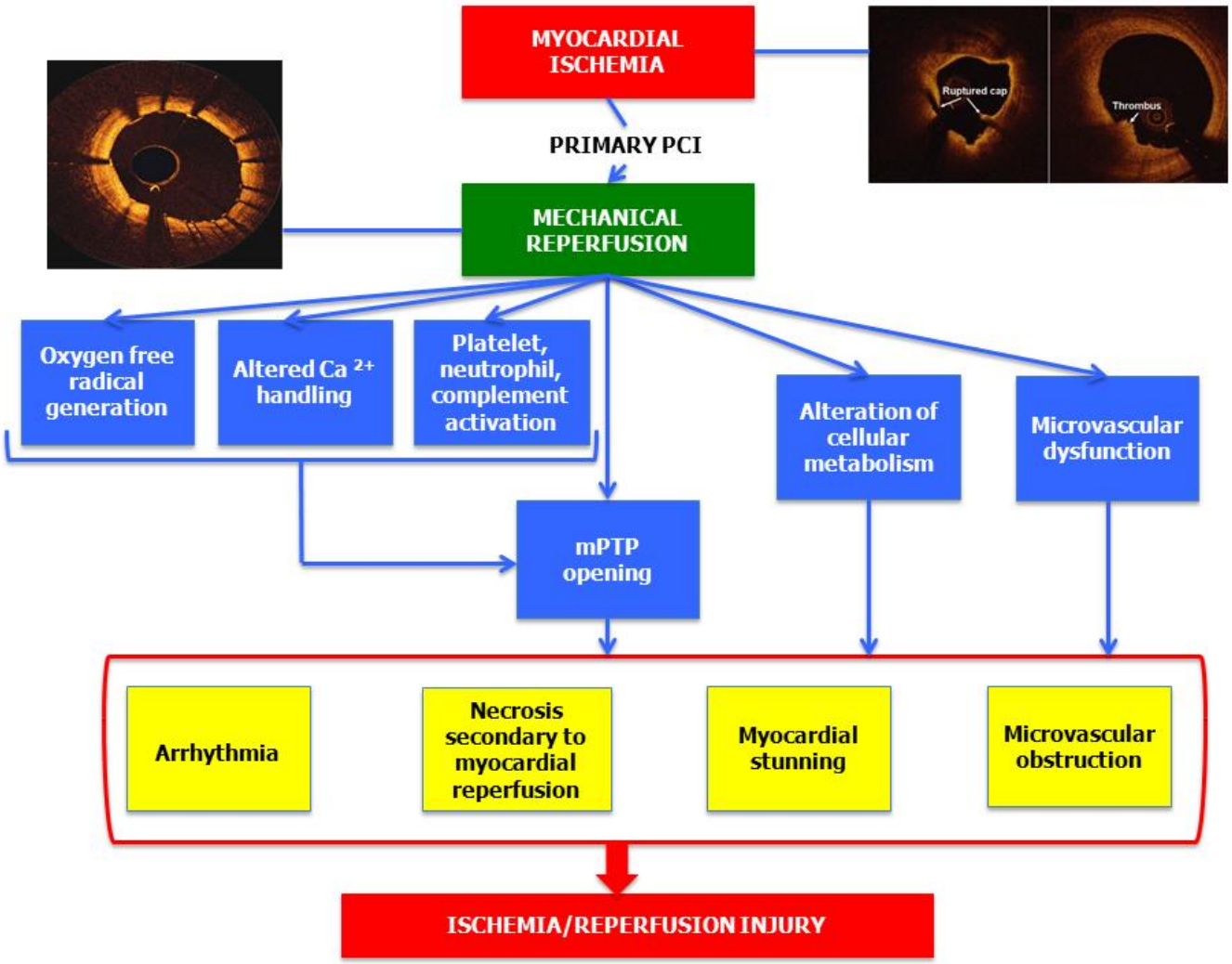
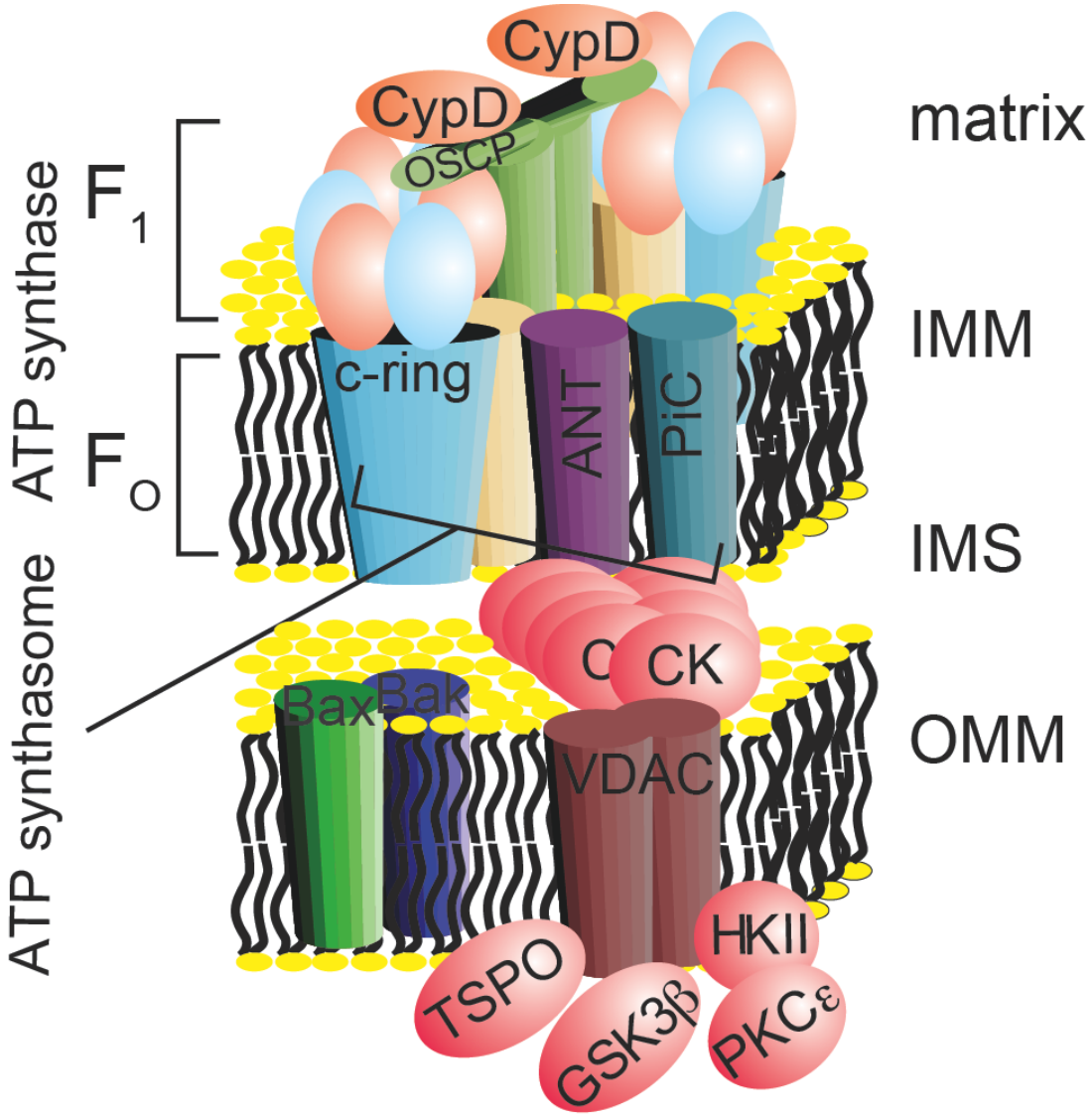


Figure 2. Novel model for mPTP structure.



The present model for mPTP is built around F1/FO ATP synthase superstructures (involving the ANT and PiC) that directly interact with the main mPTP regulator CypD. The c-ring of the ATP synthase acts as the pore of the mPTP. The model spans from the inner mitochondrial membrane (IMM) to the outer mitochondrial membrane (OMM) by interactions with the VDAC, Bax and Bak, and CK oligomers in the intermembrane space (IMS). Finally, the complex is surrounded by regulatory elements, as protein kinase C epsilon (PKCε), glycogen synthase kinase 3-beta (GSK3-β) and mitochondrial translocator protein (TSPO) are involved.

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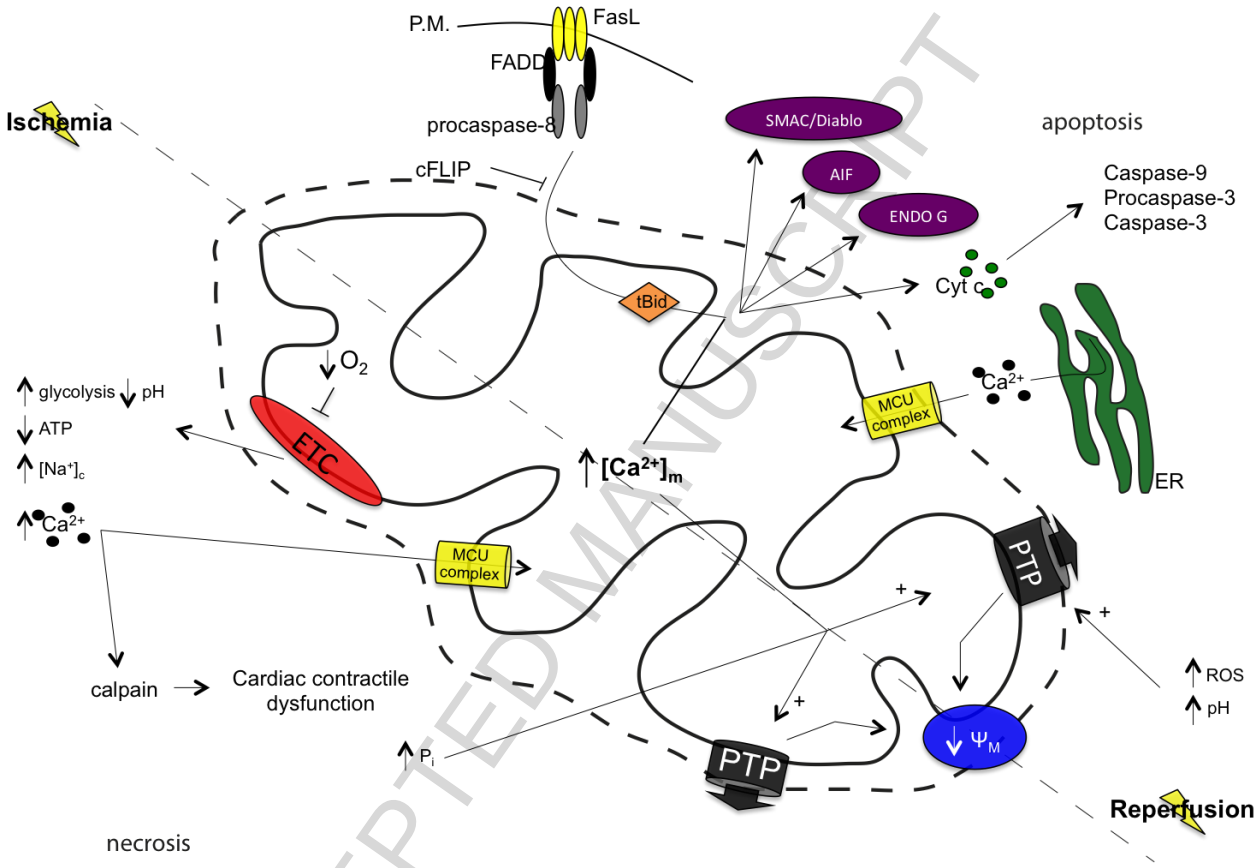
Figure 3. Mitochondrial involvement in cell death during ischemia/reperfusion injury in MI.



Ischemia is on the left side; reperfusion is on the right side. The dashed line that divides the mitochondrion reveals that both events share similar pathways that lead to different pathological effects. Ischemia: insufficient blood supply to the heart. Ischemia leads to alterations in the mitochondrial electron transport chain (ETC) complexes, anaerobic metabolism prevails as a consequence of energy failure, lactic acid accumulates, and cellular pH decreases. This accumulation of hydrogen ions causes alterations in intracellular calcium homeostasis that lead to cell death. In the heart, these cellular changes are accompanied by activation of intracellular proteases, which damage myofibrils and result in cardiac contractile dysfunction. Reperfusion: restoring blood flow. Depending on its severity, reperfusion is characterized by the increased formation of ROS, increased pH, decreased ATP production, and cell death. Some of the main pathways that occur, such as intrinsic and extrinsic apoptosis, permeability transition pore opening and lastly dissipation of the mitochondrial potential and membrane swelling, are represented in the figure.

Conflict of Interest Statement

Gianluca Campo received fee for lectures from Astrazena and Menarini.

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