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► To cite this version:

Didier Morin, Rana Assaly, Stéphanie Paradis, Alain Berdeaux. Inhibition of mitochondrial membrane permeability as a putative pharmacological target for cardioprotection.: mitochondrial membrane permeability, a pharmacological target for cardioprotection. *Current Medicinal Chemistry*, 2009, 16 (33), pp.4382-98. inserm-00433929

HAL Id: inserm-00433929

<https://www.hal.inserm.fr/inserm-00433929>

Submitted on 15 Apr 2010

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Inhibition of mitochondrial membrane permeability as a putative pharmacological target for cardioprotection

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Abstract

Myocardial ischemia-reperfusion injury is a major cause of morbidity and mortality in developed countries. To date, the only treatment of complete ischemia is to restore blood flow; thus the search for new cardioprotective approaches is absolutely necessary to reduce the mortality associated with myocardial ischemia.

Ischemia has long been considered to result in necrotic tissue damage but the reduction in oxygen supply can also lead to apoptosis. Therefore, in the last few years, mitochondria have become the subject of growing interest in myocardial ischemia-reperfusion since they are strongly involved in the regulation of the apoptotic process. Indeed, during ischemia-reperfusion, pathological signals converge in the mitochondria to induce permeabilization of the mitochondrial membrane. Two classes of mechanisms, which are not mutually exclusive, emerged to explain mitochondrial membrane permeabilization. The first occurs via a non-specific channel known as the mitochondrial permeability transition pore (mPTP) in the inner and the outer membranes causing disruption of the impermeability of the inner membrane, and ultimately complete inhibition of mitochondrial function. The second mechanism, involving only the outer membrane, induces the release of cell death effectors. Thus, drugs able to block or to limit mitochondrial membrane permeabilization may be cytoprotective during ischemia-reperfusion. The objective of this review is to examine the pharmacological strategies capable of inhibiting mitochondrial membrane permeabilization induced by myocardial ischemia-reperfusion.

MESH Keywords Adenosine Triphosphatases ; metabolism ; Apoptosis ; Mitochondrial Membrane Transport Proteins ; antagonists & inhibitors ; metabolism ; Mitochondrial Membranes ; drug effects ; metabolism ; Myocardial Reperfusion Injury ; drug therapy ; prevention & control ; Permeability ; bcl-2 Homologous Antagonist-Killer Protein ; metabolism

Author Keywords mitochondria ; heart ; ischemia-reperfusion ; necrosis ; apoptosis ; mitochondrial membrane permeability.

Introduction

Myocardial ischemia is a leading cause of death in developed countries. After an acute myocardial infarction, the only available treatment to reduce infarct size and to improve the clinical outcome is to restore blood flow (reperfusion) to the ischemic myocardium by thrombolysis, primary percutaneous coronary intervention or cardiac surgery. It is now well-known that reperfusion can induce injury and is at least as deleterious as the oxygen deficit (for a complete review on the mechanisms of the disease see [1]).

Cell death during myocardial ischemia-reperfusion has been assumed to occur primarily by necrosis. However, several studies indicate now that cell death following myocardial ischemia has features of apoptosis [2,3]; autophagy has also been suggested to play a role in myocardial ischemia-reperfusion injury [4,5]. Given that recent data indicate that the different forms of cell death are regulated and are probably interrelated [6,7], the better strategy to develop cardioprotective agents is not to define the precise mode of cell death and its proportion occurring during ischemia-reperfusion, but to identify mediators active in all forms of cell death. In this context, the mitochondrion has emerged as a relevant candidate. More importantly, the change in mitochondrial membrane permeability appears to be a major regulator of both necrotic and apoptotic cell death; the mode of death likely depends on the severity of the insult and on the ability of the cell to maintain ATP synthesis.

In this review, we will examine the role of mitochondria during myocardial ischemia-reperfusion and we will show that pharmacological inhibition of mitochondrial membrane permeability represents a relevant strategy to protect the myocardium from ischemia-reperfusion. We will address this new pharmacological concept of cardioprotection using two approaches:

- The pharmacological strategies acting directly on the mitochondrial membrane by targeting mitochondrial channels (section 3).
- The use of pharmacological agents acting indirectly on mitochondrial membrane permeability, by preventing conditions promoting membrane permeabilization (e.g., oxidative stress or by stimulation of the survival pathways referred to as the “reperfusion injury salvage kinases (RISK)” pathways [8]) (section 4).

Mitochondrial dysfunctions are at the crossroad of the deleterious events caused by myocardial ischemia-reperfusion

Ischemia results in insufficient oxygen availability for mitochondrial respiration and oxidative phosphorylation and, rapidly leads to interruption of aerobic ATP synthesis (Fig. (1) and for extensive information concerning the cellular and mitochondrial events occurring during cardiac ischemia see reviews [9–12]). The rapid depletion of intracellular creatine kinase and the concomitant increase in inorganic phosphates (Pi) stimulate anaerobic ATP generation with an increase in glycolysis and lactate production. This temporarily supplies the deficiency of the aerobic pathway in the first period of the ischemic process. When hypoxic conditions continue, the intracellular acidification induced by lactate production and the hydrolysis ATP give rise to activation of the Na^+/H^+ exchanger (as the cell tries to restore intracellular pH), Na^+/K^+ ATPase inhibition (because of the drop in ATP levels) and activation of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (due to the increase in intracellular Na^+ concentration). This results in a profound ionic imbalance, cell membrane depolarization and accumulation of cytosolic Ca^{2+} (with depletion of the sarcoplasmic reticulum Ca^{2+}). Elevated cytosolic Ca^{2+} concentrations may contribute to cellular damage by activation of degrading enzymes such as phospholipases, proteases and nucleases, cumulating in the destruction of the membrane integrity and leading to cell death if the ischemic period is of sufficient duration.

Mitochondria are initially able to buffer cytosolic Ca^{2+} by storing the excess, but this process is limited since mitochondria become at least partly de-energized, inhibiting the Ca^{2+} uniporter, the main route of entry of Ca^{2+} into mitochondria. However, during prolonged ischemia, Ca^{2+} entry may occur via reversal of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger in the heart [12]. Temporally, the maintenance of a low mitochondrial Ca^{2+} concentration associated with the cytosolic acidosis and a high concentration of ADP prevents the opening of the mitochondrial transition pore, and thus protects mitochondrial membrane impermeability and potential. If the ischemic event continues, it may contribute to myocardial damage during reperfusion, since the electron transfer chain is altered, resulting in the increased production of superoxide anions from complexes I and III. This sets the stage for an increase in reactive oxygen species (ROS) production during reperfusion [13]. In addition, the F_1F_0 -ATP synthase now hydrolyses ATP. Jennings et al. [14] established that 35% of ATP utilization observed during the first 90 minutes of complete ischemia in the canine heart is due to F_1F_0 -ATPase activity. Mitochondria are believed to hydrolyse ATP under conditions of oxygen deficit to maintain the mitochondrial membrane potential. However, this effect destroys any available ATP and favours Ca^{2+} accumulation, which may contribute to the reduced performance of the heart (stunning) during reperfusion.

Some reports have also observed decreases in NADH dehydrogenase and adenine translocase activities, a reduction of the protective mechanisms against oxygen toxicity, and decreases in superoxide dismutase (SOD) activity and glutathione content. The final extent of the damages depends on the duration and the severity of ischemic event, which emphasises the crucial time for the onset of the reperfusion (which can be combined with a pharmacological intervention). This is a critical juncture which may limit or even reverse at least some of the changes in mitochondrial functions.

Ischemic damage can paradoxically be amplified by reperfusion that corresponds to the restoration of oxygenated blood flow to the ischemic tissue. This is mainly due to the generation of ROS, which can result from the reactivation of respiratory complexes blocked in a reduced state without functional coordination (inefficient transfer of electrons generating superoxide anions). ROS have direct effects on the respiratory chain components, resulting in decreased efficiency of oxidative phosphorylation; complex I appears to be especially sensitive to ischemic injury [13, 15]. Changes in complexes III and IV were also observed, but this would occur later during the deleterious process [11]. They also cause inhibition of the enzymes of the Krebs cycle (i.e. aconitase) [16, 17]. ROS cause non-specific damages to lipids, proteins and mitochondrial DNA, and induce peroxidation of cardiolipin, a major constituent of the inner membrane, which increases the inhibition of oxidative phosphorylation [18, 19].

ROS production is exacerbated by the high concentrations of Ca^{2+} , which is driven from the cytosol into the mitochondria by restoration of the mitochondrial membrane potential. These conditions favour an increase in mitochondrial membrane permeability and the induction of the mitochondrial permeability transition pore (mPTP), which cause swelling, collapse of membrane potential, and ultimately total inhibition of mitochondrial functions [10]. This phenomenon is now considered to play a central role in different kinds of cell death; Crompton et al. [20] were the first to report that mPTP opening plays a crucial role in myocardial ischemia-reperfusion injury. It is generally accepted that mPTP opening is associated with postischemic reperfusion [21] but some studies have suggested that mPTP opening might also occur during ischemia [22]. It should be noted that mPTP is not the only mechanism by which mitochondrial membranes can be permeabilized, and the search for cardioprotective agents must consider all the mitochondrial targets that can prevent mitochondrial membrane permeabilization.

Pharmacological strategies acting by direct inhibition of mitochondrial membrane permeability by targeting mitochondrial channels

During ischemia-reperfusion, cell death occurs by necrosis and apoptosis, both being induced by the change in mitochondrial membrane permeability. Mitochondrial membrane permeability is regulated by stress signals originating in mitochondria but also those of cytosolic origin such as proteins of the Bcl-2 family. Two classes of mechanisms (Fig. (2)), which are not mutually exclusive, have been proposed to explain mitochondrial membrane permeabilization (for review see [23]).

The first one involves the participation of both the inner and the outer membranes and corresponds to the mPTP. The second mechanism involves only outer mitochondrial membrane and the formation of channels across the membrane.

Although there is a controversy concerning the structure, the regulation and the definite role of these different channels, strong evidences indicate that proteins of the Bcl-2 family contribute to both mechanisms [24 ,25].

Triggering mitochondrial membrane permeabilization induces the release of cell death effectors and the loss of mitochondrial functions which are fundamental for cell survival. Thus, drugs able to block or to limit mitochondrial membrane permeabilization might be cytoprotective during ischemia-reperfusion and this pharmacological approach is reviewed in this section (Fig. (3)).

The mitochondrial transition pore as a pharmacological target

The mitochondrial respiratory chain catalyzes the oxidation of substrates by oxygen and couples electron flux to H^+ pumping to establish an H^+ electrochemical gradient across the inner membrane. ATP is synthesized by the enzyme F_1F_0 -ATP synthase which utilizes the free energy released by the H^+ backflow from intermembrane to mitochondrial matrix. Therefore, the maintenance of the H^+ gradient and the impermeability of the inner membrane to ions, especially to H^+ , is vital to the cell. However, under stress conditions, the permeability of the membrane may increase with the formation of a voltage-dependent nonspecific pore, known as the mPTP, which allows the influx of water and molecules up to 1.5 kD and therefore induces swelling of the organelle matrix. An extensive swelling results in the rupture of the outer membrane and in the release of proapoptotic proteins into the cytosol. The exact composition of this pore remains uncertain although it was formerly thought to include adenine nucleotide translocase (ANT) modulated by cyclophilin D (CyP-D) in the inner membrane and a voltage-dependent anion channel (VDAC) in the outer membrane [10 ,26]. CyP-D is a member of the cyclophilin family which displays peptidyl-prolyl cis-trans isomerase activity and resides in the mitochondrial matrix. During mPTP formation, CyP-D interacts with the inner membrane and is thought to induce a conformational change of ANT, leading to an increase in inner membrane permeability (Fig. (2)).

In vivo experiments confirm the role of CyP-D-dependent mitochondrial mPTP for mediating Ca^{2+} - and oxidative damage-induced cell death: CyP-D-deficient mice are protected from ischaemia-reperfusion-induced cell death in vivo , whereas CyP-D-overexpressing mice show mitochondrial swelling and spontaneous cell death [27 –29]. These genetic experiments also provided significant information about the mechanism of cell death triggered by mPTP. CyP-D-deficient cells responded to various apoptotic stimuli, but showed resistance to necrotic cell death induced by ROS and Ca^{2+} overload. This led to the conclusion that CyP-D and thus mPTP do not play a major role in the apoptotic process, but rather are major components of the necrotic death pathway.

Studies on mitochondria lacking these putative components of the mPTP did not permit definit conclusions about the identity of its membrane components. Indeed, a functional mPTP can form in the absence of ANT in mice [30], and it was suggested that ANT may not be the protein which binds CyP-D [31]. Juhaszova et al. [32] suggest that ANT may be a non-essential structural component of the mPTP but may have a regulatory role in mPTP induction (see Fig. (2A)). VDAC also may not be an essential component of the mPTP as the pore can be induced in mitochondria devoid of all VDAC isoforms [33]. It should be added that other proteins such as hexokinase, creatine kinase or the mitochondrial translocator protein (TSPO), have been proposed to be involved in pore formation, but to date it is not known whether these proteins are structural or regulatory components of the pore.

Taken together, these data show that the molecular composition of mPTP remains a source of debate, but nevertheless, strong evidence suggests that mPTP contributes to cellular injury during myocardial ischemia-reperfusion: (1) mPTP opening is favored by conditions prevailing during ischemia-reperfusion (2) mPTP might control cell death by releasing apoptogenic factors [10 ,34 ,35], (3) mPTP is regulated by the pro- and the anti-apoptotic members of the Bcl-2 family [36 ,37], (4) increasing lines of evidence suggest that mPTP is involved in the cardioprotective effect of ischemic pre- and post-conditioning (see section 4.1.).

Therefore, targeting mPTP appears as a promising pharmacological approach in ischemia-reperfusion and numerous studies show that almost any procedure that reduces mPTP opening affords protection against ischemia-reperfusion injury.

The first demonstration of the relevance of this approach was provided by the immunosuppressant agent cyclosporin A (CsA) which binds to CyP-D with high affinity and precludes the interaction of this protein with a component of the mPTP, thus inhibiting its opening. CsA protected against hypoxia-reoxygenation-mediated cell death in isolated cardiomyocytes [38 ,39] and reduced infarct size in ex-vivo models of cardiac ischemia-reperfusion [40 ,41]. Shanmuganathan et al. [42] have also demonstrated that inhibition of mPTP opening by

CsA at the onset of reoxygenation protected the human myocardium against lethal hypoxia-reoxygenation injury. The clinical use of CsA is hampered by pharmacokinetic and pharmacodynamic parameters [43] and by the fact that CsA fails to inhibit pore opening when mitochondria are exposed to strong stimuli [44]. However, a recent clinical study showed that an intravenous bolus of 2.5 mg/kg CsA just before reperfusion reduced infarct size in patients suffering from acute myocardial infarction [45].

Similar cardioprotective effects were obtained with non-immunosuppressant derivatives of CsA, such as [N-methyl-ala⁶]CsA, [N-methyl-Val⁴]CsA, Debio 025 or NIM811, [21 ,40 ,46 ,47]) confirming the relevance of this approach. Sanglifehrin A also prevents myocardial ischemia-reperfusion injury [48 ,49]. This drug is unrelated to CsA but potently inhibits mPTP by inhibiting the peptidyl-prolyl cis-trans isomerase activity of CyP-D (Fig. (3) and (4)).

It should be added that the inhibition of mPTP opening by CsA was also effective in a particular case of ischemia-reperfusion, the hypothermic preservation of the heart before transplantation. Indeed, CsA improved functional recovery after long-time hypothermic heart preservation [50].

Although the molecular structure of mPTP remains unknown, other approaches have been developed to find new mPTP inhibitors. Binding sites involved in the regulation of mPTP were described [51]. Clinically approved drugs, including tricyclic antidepressants, antipsychotics and antiarrhythmic agents were found to bind to these sites and to inhibit mPTP in vitro [51 ,52]. Some of these drugs reduced neurological impairment in mice subjected to middle cerebral artery occlusion/reperfusion [52], but, to our knowledge, have not been tested in myocardial models of ischemia-reperfusion. Most of these drugs are amphiphilic cations which are known to interact with biological membranes which could change mitochondrial membrane potential and thus possibly induces closure of the pore [53].

Another approach to design new pore inhibitors was provided by the results of Bernardi and colleagues who demonstrated that the coenzyme Q family in addition to its antioxidant effects and its role in the regulation of electron transfer modulates mPTP opening by acting on a specific site [54]. Exogenous coenzyme Q₀ (ubiquinone 0) and coenzyme Q₁₀ (decylubiquinone) are very potent inhibitors of mPTP opening induced by Ca²⁺ overload, whereas coenzyme Q₁ (ubiquinone 5), which did not inhibit pore opening per se, counteracted the effects of coenzyme Q₀ and decylubiquinone [54]. As a putative component of mPTP, VDAC was suggested to be one of the target of ubiquinone analogues [55], but since liver mitochondria from mice lacking VDAC1 exhibit normal mPTP opening and inhibition by ubiquinone analogues [56], this hypothesis seems unlikely. It should be noticed that the design of new mPTP inhibitors from coenzyme Q may be difficult since minor structural changes profoundly modify the effects of quinones on the mPTP [57].

To our knowledge, it has not been established whether this is what is responsible for the antiischemic effect of coenzymes Q [58] and data indicate that coenzyme Q₀ even impairs myocardial performance following ischemia-reperfusion [44]. However, the antioxidant properties of coenzyme Q may contribute to mPTP inhibition as decylubiquinone was shown to inhibit redox-activated permeability transition [59].

Taken together, these data suggest that the direct inhibition of mPTP opening constitutes a relevant objective in cardioprotection. This requires the search for new direct mPTP inhibitors [55 ,60] with good specificity and selectivity and the identification of the molecular site of action of these compounds to further elucidate mPTP structure.

The MAC/Bak channel

It is now well-established that mPTP is not the only mechanism by which the mitochondrial membrane can be permeabilized [61]. Indeed, cytochrome c release was observed in the absence of mitochondrial depolarization [62] and without loss of mitochondrial outer membrane integrity (i.e., independently of mPTP opening), indicating that permeabilization of the outer membrane is a regulated phenomenon occurring via the formation of a pore [24]. This type of release has been observed during cardiac and liver ischemia [63 –65].

Mitochondrial membrane permeabilization is under the control of the Bcl-2 family of proteins (for a recent review see [66]). Proapoptotic members (e.g. Bax, Bak or the BH3-only subfamily proteins Bid, Bad, Bnip3) facilitate membrane permeabilization and promote the release of cytochrome c and other intermembrane space components. Cytochrome c triggers formation of the apoptosome complex and activation of caspase-9 [61]. This is not the only pathway which can be activated by outer membrane permeabilization, since two other proteins, apoptosis inducing factor (AIF) and endonuclease G, which are also released after outer membrane permeabilization, can induce apoptosis in a caspase-independent manner [67 ,68]. Two main possibilities have been proposed to be responsible for governing this increase in permeability. Bax resides in the cytosol in normal, healthy cells but, once activated, translocates to mitochondria, where 1) it incorporates into the outer membranes and oligomerizes to form permeable pores [3 ,61] and/or 2) interacts with existing channels such as VDAC [3 ,69]. The activation of Bax is regulated by the BH3-only proteins, which facilitate Bax channel formation. It should be pointed out that the Bax channel appears to be identical to the mitochondrial apoptosis-induced channel (MAC channel; [24]), which was first detected on mitochondria in patch-clamp experiments. The anti-apoptotic members Bcl-2 and Bcl-x_L are mainly localized to the mitochondrial outer membrane where they antagonize the pro-apoptotic effect of Bax and Bak [70].

The importance of the Bcl-2 family proteins in myocardial ischemia-reperfusion injury was demonstrated in mouse models. Indeed, Bax deletion in mice, interference with Bax activation, or over-expression of Bcl-2 levels have been shown to attenuate apoptosis and reduce infarct size, while reduction of Bcl-2 levels (via antisense oligonucleotides) suppressed protection against injury [72–76]. Therefore, anti-apoptotic interventions targeting Bcl-2 family proteins provide opportunities for possible antiischemic therapies [77], either through a gain of antiapoptotic function or loss of proapoptotic function. Polster et al. [78] demonstrated that the well-known drugs propranolol and dibucaine inhibited Bax-induced permeability changes and cytochrome c release from mitochondria through a direct interaction with the lipid membrane. A similar approach allowed others to identify 3,6-dibromocarbazole piperazine derivatives of 2-propranolol as inhibitors of cytochrome c release triggered by induction of the Bax channel [79]. In the same way, a pentapeptide derived from the Bax-modulating Ku70 protein inhibited Bax-dependent apoptosis [80]. More recently, Hetz et al. [81] identified two blockers of Bax channel activity, bci1 and bci2 (Fig. (4)), which prevented cytochrome c release *in vitro* and *in vivo*, and protected neurons in a gerbil model of global brain ischemia at reperfusion. It is a promising pharmacological strategy, but to our knowledge, these compounds have not been evaluated during myocardial ischemia.

BH3-only Bcl-2 proteins

The activation of Bax and Bak is regulated by the class of Bcl-2 proteins that contain single BH3 domains. These proteins control apoptotic signals upstream of mitochondria and transmit them directly or indirectly to Bax and Bak [66], thus they represent specific targets for preventing myocardial ischemia-reperfusion injury. This appears to be an excellent point of intervention because only activation of Bax and Bak would be affected, minimizing cell apoptosis. This approach would also facilitate maintenance of mitochondrial integrity.

Among these proteins Bid (BH3-Interacting Domain death agonist) is one of the most abundant in mammalian tissues, including the heart [82]. Bid is subjected to a protein cleavage by caspase-8, granzyme, or calpain, and truncated Bid (tBid) translocates to the mitochondria where it is involved in Bax/Bak activation and/or Bax/Bak channel formation [66]. Thus, Bid is at the crossroad of the intrinsic and the extrinsic death pathway. Bid-mediated apoptosis has been shown to contribute to myocardial ischemic injury [83] and Bid-deficient mice have reduced infarct size and improved cardiac functions after ischemia-reperfusion [3]. In addition, calpain inhibitors reduce infarct size in animal models of isolated perfused heart [84] and slow the progression of heart failure in rats [85,86]. These data suggest that Bid is an attractive target during myocardial ischemia-reperfusion, and pharmacological inhibitors which are effective *in vitro* assays have already been designed [87].

Other BH3-only Bcl-2 proteins have been implicated in cardiac ischemia-reperfusion injury [88]. Bnip3 (Bcl-2/adenovirus E1B 19kDa interacting protein 3) has been shown to mediate mitochondrial dysfunction and cell death of ventricular myocytes subjected to hypoxia [89–90]. It was also reported to contribute to myocardial ischemia-reperfusion injury through a mitochondrial death pathway [91–92]. Interestingly, a TAT-fusion protein encoding a carboxyl terminal transmembrane deletion mutation in of Bnip3 conferred protection against myocardial ischemia-reperfusion injury, improved cardiac function, and protected mitochondrial integrity [92], confirming that Bnip3 could constitute a relevant therapeutic target.

In the same way, Puma (p53-Upregulated Modulator of Apoptosis) was upregulated in response to hypoxia/reoxygenation in isolated cardiomyocytes and Puma-deficient mice have reduced infarct size and improved cardiac function after myocardial ischemia-reperfusion [93]. Until now, pharmacological strategies targeting selectively Puma have not been described.

The pharmacological strategy inhibiting mitochondrial outer membrane permeability, and thus apoptosis, by manipulation of the Bcl-2 family proteins to protect the myocardium against ischemia-reperfusion is very recent. It has already provided interesting results, which support the idea that a clinical benefit might be obtained in the near future.

The voltage-dependent-anion channel (VDAC) as a pharmacological target

Another potential mechanism for outer mitochondrial membrane permeabilization might involve VDAC which is the major permeability pathway for metabolites through the mitochondrial outer membrane [94]. Different isoforms of VDAC have been identified [95,96] and the role of VDAC in cell death induced by mitochondria seems to be isoform specific. Indeed, VDAC1 has been implicated in the mitochondrial release of proapoptotic protein whereas VDAC2 displays antiapoptotic properties [97,98].

It is important to note that much of the evidence involving VDAC opening and closing in proapoptotic protein release has arisen from *in vitro* experiments, and it is not clearly established whether this can be observed *in vivo* [99]. However, VDAC is a hypothetical component of mPTP, interacts with pro- and anti-apoptotic proteins of the Bcl-2 family members, and VDAC has been also proposed to control the release of cytochrome c without mPTP opening [97,100,101].

Taken together, these data make VDAC a putative target in ischemia-reperfusion. Interestingly, a strategy developed to inhibit VDAC was shown to reduce myocardial ischemia-reperfusion injury. Perfusion of rat hearts with a cell permeable peptide corresponding to the BH4 domain of Bcl-X_L, which had been reported to close VDAC and to prevent the VDAC-mediated release of cytochrome c [102], was

shown to inhibit creatine kinase release and to reduce myocardial cell death [84]. In the same way, the Ca^{2+} -dependent actin-regulatory protein gelsolin was suggested to inhibit apoptosis by blocking mitochondrial VDAC activity [103]. This is in disagreement with recent results that indicate that closure of VDAC, not opening, leads to mitochondrial outer membrane permeabilization and apoptosis [104]. It is clear that a better knowledge of the role of VDAC, if any, in ischemic injury, will take time, given the lack of proven pharmacological compounds specifically targeting VDAC.

Mitochondrial potassium (mitoK_{ATP}) channels

MitoK_{ATP} channels were first identified in liver mitoplasts (mitochondria stripped of the outer membrane) by Inoue et al. [105] in patch-clamp experiments, and then by light scattering experiments in liver and heart isolated mitochondria [106–107]. Evidence for the involvement of mitoK_{ATP} channels in cardioprotection was provided by the observation that mitoK_{ATP} openers, like diazoxide or nicorandil, can protect the myocardium against ischemia-reperfusion injury [108–110] and mimic ischemic pre-conditioning, while blockers inhibit [111–113]. In the same way, recent data show that the myocardial protection conferred by ischemic post-conditioning is blocked by the mitoK_{ATP} channel blocker 5-hydroxydecanoate and thus achieved at least in part by opening of mitoK_{ATP} channels [114]. The mechanism for mitoK_{ATP} channels opening during pre- and post-conditioning is currently the subject of intense investigation.

Several recent studies have linked the protein kinase C epsilon (PKC ϵ), which translocation to mitochondria seems important for pre-conditioning [115], to mitoK_{ATP} channel opening. Other kinases have also been involved [116–117].

The mechanism by which activation of mitoK_{ATP} results in cardioprotection is not well-understood, but implies a limitation in mitochondrial membrane permeability. Several hypotheses have been proposed. The first one considers that the beneficial effect of mitoK_{ATP} activation could be the consequence of a partial dissipation of the membrane potential caused by the net influx of K^+ [118]. This would reduce Ca^{2+} entry or release an excess of Ca^{2+} , preventing Ca^{2+} overload [119] and thus mPTP opening during reperfusion. This is consistent with the results of Korge et al. [120] and Facundo et al. [121], who reported that diazoxide prevents opening of the mPTP induced by elevated Ca^{2+} concentrations in isolated mitochondria. However, this hypothesis has been disputed, because mitochondrial membrane depolarization generated by K^+ flux does not appear to be sufficient to affect Ca^{2+} transport [122] and has not been observed in intact cardiac myocytes [123]. Moreover, a higher depolarization may lead to deleterious phenomena more frequently associated with cell death, than t protection (such as decreased ATP synthesis and even mPTP opening).

The second hypothesis proposes that perturbed ROS production is responsible for mitoK_{ATP} channel effect. Depending on the conditions, pre-conditioning phase, ischemia or reperfusion, mitoK_{ATP} channel openings could either increase or attenuate ROS generation [121, 124], but the link between mitoK_{ATP} channels, ROS and the induction of a cytoprotective effect is not clear. In this context, Hausenloy et al. [125] suggested that the cardioprotection induced by diazoxide is ROS-dependent and due to transient mPTP opening-mediated ROS release during the preconditioning phase. In this scheme, mPTP opening may act as a protective mechanism in response to different stimuli during the pre-conditioning period to inhibit its deleterious opening during the reperfusion phase. This is an attractive mechanism but is still highly controversial [126]. Similarly, Costa et al. [127] have reported that mPTP inhibition by mitoK_{ATP} opening requires the generation of ROS.

The third hypothesis, which is not mutually exclusive, considers the only well-defined physiological function of mitochondrial K^+ transporters, i.e. the regulation of the matrix volume [128]. Dos Santos et al. [129] hypothesized that opening of the mitoK_{ATP} during ischemia resulted in a slight mitochondrial swelling which preserved the structure of the intermembrane space and thus maintained VDAC in a low permeability state for adenine nucleotides. This would reduce glycolytic ATP entry into mitochondria and, therefore, ATP hydrolysis by F_1F_0 -ATPase, and would preserve ADP for phosphorylation upon reperfusion (for review see [130]). Thus, the mechanism by which mitoK_{ATP} channels exert their cardioprotective effect is still a source of debate, but most of the studies suggest that opening of mitoK_{ATP} channels prevents cellular death by decreasing mitochondrial ROS release and Ca^{2+} overload during the reperfusion, thus inhibiting mPTP.

Based on these experimental data, clinical studies have been performed. Some of them reported cardioprotection with intravenous administration of nicorandil (a mixed mitoK_{ATP} channels opener and nitric oxide donor) in patients with acute myocardial infarction [131, 132], but another study failed to demonstrate this effect in the same clinical setting [133].

However, the full demonstration of the involvement of mitoK_{ATP} channels in cardioprotection is hampered by the absence of selective agents for the mitoK_{ATP} channel; the mitoK_{ATP} channel openers and inhibitors which have been used to demonstrate the cardioprotective effects of these channels (most notably diazoxide and 5-hydroxydecanoate), are claimed to be specific for this channel, but numerous studies argue against this specificity (see the review of Halestrap et al. [134]).

A better understanding of the cellular mechanisms and of the role of $\text{mitoK}_{\text{ATP}}$ channels will require the development of more specific and more selective pharmacological entities; however, they still appear to be a promising target for the development of new cardioprotective agents.

The mitochondrial translocator protein (TSPO)

TSPO (formerly known as Peripheral Benzodiazepine Receptor [135]) is a ubiquitous high affinity cholesterol binding 18 kDa protein primarily located on the outer mitochondrial membrane, where it is associated with VDAC [136]. It has been implicated in numerous biological functions [135], including regulation of mPTP opening [136, 137] but the exact physiological role of TSPO remains unclear (except in steroid-producing tissues where TSPO mediates the transport of cholesterol from the outer to the inner mitochondrial membrane and promotes pregnenolone synthesis [138]). TSPO is also present in non steroidogenic tissues, especially in the heart, where its function is unknown. Several reports suggest that TSPO ligands might modulate apoptotic responses [139] and play an anti-apoptotic role in oxidative stress conditions such as ischemia-reperfusion [140]. In this context, TSPO expression correlated with the quality of kidney preservation, indicating that it might serve as an index of kidney and mitochondrial viability during storage [141]. Thus, one means to improve resistance of the cells to oxidative stress generated during ischemia would be to increase TSPO expression levels using pharmacological tools to increase TSPO transcription. Leducq et al. [142] found that the irreversible TSPO ligand SSR180575 prevented the cellular damages resulting from oxidative stress and cardiac injuries induced by ischemia-reperfusion in rodents, and Obame et al. [143] showed that the specific TSPO ligand 4'-chlorodiazepam (Fig. (4)) reduced infarct size in both global and regional models of myocardial ischemia-reperfusion in rats. The protective mechanism involved limiting mitochondrial membrane permeabilization, notably a resistance of mitochondria to mPTP opening, associated with a reorganization of the balance between pro- and anti-apoptotic proteins of the Bcl-2 family proteins at the level of mitochondrial membranes [143]. Taken together, these data demonstrate a role for TSPO as a modulator of necrotic and apoptotic cell death induced by ischemia-reperfusion, suggesting that TSPO could constitute a possible target for cardioprotection.

Pharmacological strategies acting indirectly on mitochondrial membrane permeability

Ischemic pre- and post-conditioning

Ischemic pre-conditioning (IPC) is one of the more powerful processes to protect the heart from lethal ischemia-reperfusion injury. IPC was originally described by Murry et al. [144], who demonstrated that a sequence of successive short periods of ischemia reduced infarct size produced by a subsequent long ischemic period. Studies revealed that IPC caused the release of G protein-coupled receptors agonists such as adenosine, bradykinin or opioids that activated a cascade of cardioprotective kinases referred to as "Reperfusion Injury Salvage Kinase" (RISK) pathway [145]. This cascade includes PKC, the PI3kinase-AKT pathway, the ERK1/2 pathway, and downstream proteins such as glycogen synthase kinase-3 β (GSK-3 β), PKG, Bad or endothelial nitric oxide synthase (eNOS). PKC ϵ is a primary cardioprotective PKC isoform, whereas PKC δ promotes injury. Activation of AKT and ERK1/2 and phosphorylation of GSK-3 β appear essential for IPC in numerous studies, although this has been questioned by Nishino et al. [146]. More recently, Zhao et al. [147] demonstrated that brief repetitive ischemic periods of ischemia followed by reperfusion after a lethal ischemic period results in a robust reduction in infarct size, termed ischemic post-conditioning (IPOC). Subsequent studies have shown that IPC and IPOC use a similar signalling pathway, i.e. the RISK pathway, and that classical ligands able to trigger IPC, such as adenosine or opioids, were also able to induce IPOC [148]. For a detailed characterization of the signalling pathways involved in IPC and IPOC see [115]. From a clinical point of view the discovery of IPOC is fundamental as it can be applied in patients [149].

The cellular mechanisms governing IPC and IPOC protection are not fully understood, but increasing lines of evidence suggest that indirect inhibition of mitochondrial membrane permeability, through inhibition of mPTP opening during reperfusion, may be the target of the RISK pathway [150–153]. The mechanism by which the RISK pathway inhibits mPTP is unclear, but several candidates have emerged which may act in concert to mediate mPTP inhibition (Fig. (5)). These include:

- eNOS generation of NO, which alone [154], or through the PKG-PKC ϵ - $\text{mitoK}_{\text{ATP}}$ channel can limit mPTP opening [115, 127],
- phosphorylation and/or complex formation with cytosolic proteins like PKC ϵ can interact with mPTP [155, 156], whereas the activity of PKC δ and its translocation to mitochondria at the onset of reperfusion mediates apoptosis [157],
- phosphorylation and inhibition of GSK-3 β , a downstream target of AKT, which have been demonstrated to inhibit mPTP [150, 153]. Nishihara et al. [158] suggested that the binding of GSK-3 β to ANT might be responsible for mPTP inhibition and the myocardial protection afforded by IPC,
- inhibition of downstream signals of the RISK pathway, such as Bax translocation from cytoplasm to mitochondria [159] or hexokinase II activation [160].

However, whatever the mechanism(s) connecting the RISK pathway to mPTP inhibition, the activation of this cascade of kinases offers the opportunity to promote mPTP inhibition and thus cardioprotection at reperfusion. This property can be exploited therapeutically as described in the following studies.

Targeting of the RISK pathway by ischemic post-conditioning

IPOC was recently described, but small clinical studies have already been initiated [161 –163]. In these studies, IPOC was induced in patients by a series of successive episodes of inflation-deflation of the angioplasty balloon during percutaneous coronary intervention. IPOC significantly protected the heart against ischemia-reperfusion-induced injury and, importantly, the protective effect is of significant duration [163].

Targeting the RISK pathway via pharmacological agents

The administration of high doses of adenosine at reperfusion in acute myocardial infarction resulted in myocardial protection in two small clinical studies [164 ,165]. A reduction in infarct size was also observed in larger randomized controlled studies in which adenosine was administered as an adjunct to thrombolytic therapy after the beginning of reperfusion [166 ,167]. However, this pharmacological strategy did not improve clinical outcomes [167].

Morphine is another G protein-coupled receptor agonist which is known to activate the RISK pathway and to cause potent cardioprotection when administered upon reperfusion [168]. Recently, Obame et al. [153] have shown that morphine provides protection against myocardial infarction through inhibition of mPTP opening. Thus, the use of morphine, a well-known drug which can be administered acutely in safe conditions, as an adjunct of myocardial reperfusion, may be a useful clinical strategy in acute myocardial infarction.

It is also possible to act upstream in the RISK pathway, as several groups have shown that inhibitors of GSK-3 β reduced infarct size in mice and rats, and that this effect was associated with the inhibition of mPTP opening [153 ,168 ,169].

Similarly, beside their lipid-lowering actions, statines (3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors) have been shown to exhibit cardioprotective properties [170 ,171]. The mechanism seems to involve stimulation of several kinases in the RISK pathway but also activation of the mitoK_{ATP} channel through NO generation and inhibition of mPTP opening [172].

Another possible strategy is to directly target PKC via PKC ϵ activators or PKC δ inhibitors. A recent small clinical study of acute myocardial infarction using the PKC δ inhibitory peptide, KAI-9803, which was delivered via intracoronary injection during percutaneous coronary intervention, revealed signs of cardioprotection. Unfortunately, the observed improvements were not significant, probably because of the small size of the sample [173]

Mitochondria-targeted antioxidants in limiting mitochondrial membrane permeability

In physiological conditions, ROS are formed within mitochondria and are eliminated by powerful antioxidant systems [174]. Under pathological conditions, especially during post-ischemic reperfusion, the sudden influx of oxygen to the mitochondrial respiratory chain leads to a burst of ROS (as a superoxide anion; [175]) which can overwhelm endogenous antioxidant systems and deplete the reducing compounds protecting mitochondria against oxidative insults. It should be noted that the respiratory chain is not the only source of ROS in mitochondria [174]. For instance, MAO-A has been shown to represent an important source of hydrogen peroxide in rat heart [176]. ROS cause non-specific damage to lipids, proteins and mitochondrial DNA. When associated with mitochondrial Ca²⁺ overload and adenine nucleotide depletion, conditions that pertain during myocardial reperfusion, high levels of ROS can induce opening of the mPTP [44]. ROS generated during early reperfusion have been found to be primary activators of mPTP and cardiomyocyte death [177], and Clarke et al. [178] have reported that IPC-induced inhibition of mPTP is probably mediated by a reduction in oxidative stress. This property might also contribute to the cardioprotective effect of some therapeutic drugs such as pyruvate [179], the β -adrenoceptor blocking agent carvedilol [180] or the anaesthetic propofol, frequently used for cardiac surgery [181].

These data highlight the major role of ROS in the increase in mitochondrial membrane permeability. Thus, any antioxidant treatment is likely to protect mitochondria and ultimately the myocardium at reperfusion. Numerous pharmacological strategies have been developed to scavenge ROS or to increase mitochondrial ROS degradation [182]. Recently, Bogner et al. [183] have developed a novel superoxide dismutase mimetic drug which was able to eliminate ROS in the microenvironment of the mPTP and to inhibit its opening, leading to the protection of the myocardium against myocardial ischemia-reperfusion (Fig. (4)).

An interesting result was also obtained with the free radical scavenger edavarone which was shown to inhibit mPTP opening and to prevent cardiac reperfusion injury [184]. In a recent clinical study, edavarone delivered via intravenous injection during percutaneous coronary intervention reduced infarct size and reperfusion arrhythmias, and improved short term clinical outcomes in acute myocardial infarction [185]. However, most of the clinical studies with conventional antioxidants have yielded disappointing results [186].

Several reasons can be evoked to explain this failure; among them, the main reason may be the difficulty of delivering the drug to mitochondria in situ. To overcome these limitations, investigators have synthesized amphiphilic cations from well-established antioxidant compounds, which are sufficiently lipophilic to cross lipid bilayer membranes and which can be selectively taken up into the mitochondria by the large, negative inside, inner membrane potential [187, 188]. These drugs include alpha tocopherol (MitoVit E) and ubiquinone (MitoQ) (Fig. (4)). A recent study demonstrated that feeding rats with MitoQ significantly limited cardiac reperfusion-injury [189]; this approach might provide a promising pharmacological strategy.

Using a similar idea, a novel class of small cell-permeable antioxidant peptides (Fig. (4)) that target mitochondria have been reported [190]. Their antioxidant effect is due to the presence of basic amino acids, tyrosine and dimethyltyrosine, which are effective in scavenging ROS. These peptides enter the mitochondria in a potential-independent manner (contrary to MitoQ and MitoVitE) because they include a sequence motif targeted to mitochondria. These peptides reduce mitochondrial ROS production, inhibit mPTP opening, prevent the release of cytochrome c from mitochondria [190] and protect against myocardial ischemia-reperfusion ex vivo and in vivo [191].

Another alternative antioxidant approach would be to prevent mitochondrial ROS production to protect mitochondrial membrane. A first possibility would be the development of specific MAO-A inhibitors, since MAO-A inhibition has been shown to reduce myocardial damage induced by ischemia-reperfusion [192]. This can also be achieved by mild uncoupling [193], decreasing ROS generation by dissipating the electrochemical potential of H^+ , allowing an increase in electron flux at the expense of ubiquinol. Mild uncoupling might represent an antioxidant defense mechanism during the reperfusion period, preventing the sudden increase in membrane potential, the overproduction of ROS, and the increase in mitochondrial membrane permeability. Experimental results have confirmed the relevance of this approach. Decreased ROS formation associated with myocardial protection has been demonstrated using uncouplers [194, 195], or by overexpressing uncoupling proteins [196, 197], and the data of McLeod et al. [198] support a role for UCP2 in delayed IPC induced cardioprotection.

Concluding remarks

This manuscript highlights that mitochondria represent a pertinent target to develop cardioprotective pharmacological therapies and the crucial role of the preservation of mitochondrial membrane integrity to protect cardiac cells during myocardial ischemia-reperfusion. Beside their essential roles in energy production, Ca^{2+} homeostasis and ROS production, mitochondria control necrotic and apoptotic myocardial cell death by regulating the permeability of its membranes. Indeed, mPTP may be considered as a central event in necrosis (although a controversy persists concerning the molecular composition of mPTP), whereas the permeabilization of mitochondrial outer membranes by proteins of the Bcl-2 family leads to apoptosis. On the whole, the studies presented above reveal that both anti-necrotic and anti-apoptotic strategies targeting mitochondrial permeability are able to protect cardiomyocytes from death during ischemia-reperfusion. However, the signaling mechanisms mediating mitochondrial membrane permeabilization in ischemia-reperfusion are diverse, so it seems likely that a combination of pharmacological therapies, i.e. compounds combining different pharmacological effects or co-administration of several drugs acting simultaneously on distinct targets, should afford a better protection against myocardial ischemia-reperfusion injury.

Acknowledgements:

R. Assaly was supported by a doctoral grant from region Ile-de France and S. Paradis was supported by the Ministère de la Recherche et de la Technologie. We gratefully acknowledge Dr W. S. Neckameyer (Department of Pharmacological and Physiological Science, Saint-Louis University School of Medicine, St Louis MO, USA) for reading the manuscript.

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Fig. (1)

Scheme illustrating the mitochondrial sequence of events triggered by myocardial ischemia-reperfusion

The scheme summarizes the processes described in the text, showing the relationships between oxygen consumption, ATP production, Ca^{2+} accumulation, reactive oxygen species (ROS) generation and the initiation of cell death through activation of the mitochondrial permeability transition pore (mPTP). During ischemia, cellular acidosis along with a high concentration of ADP prevents mPTP opening although favourable conditions prevail ($\Delta\Psi_m$ decrease, high P_i , slight increase in ROS). During reperfusion, high matrix concentrations of Ca^{2+} and P_i associated with a burst of ROS favour mPTP opening despite the antagonizing effect of $\Delta\Psi_m$ recovery.

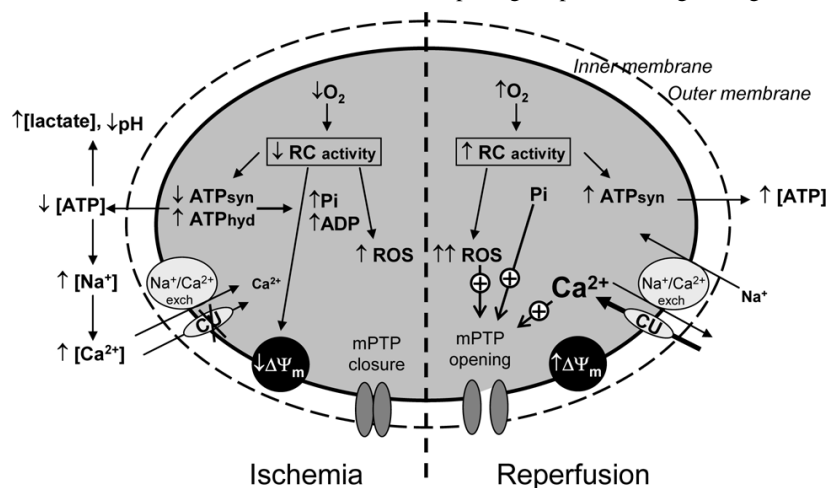
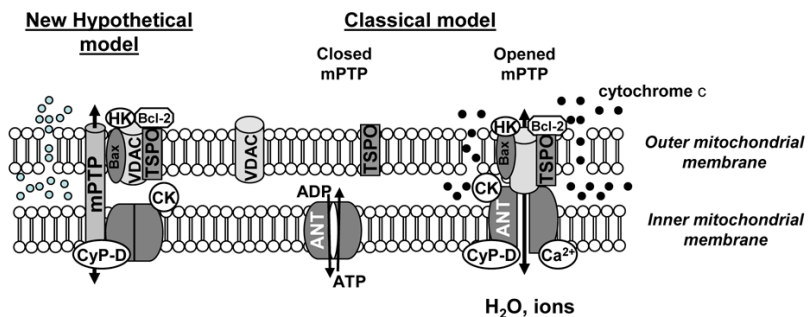


Fig. (2)

Hypothetical models of the increase in mitochondrial membrane permeability during ischemia-reperfusion

A: mitochondrial permeability transition pore (mPTP). Classical model: mPTP is formed by the interaction of VDAC, ANT and CyP-D at the contact sites between the inner and the outer membrane and can be modulated by other proteins such as creatine kinase (CK), hexokinase (HK), translocator protein (TSPO) and proteins of the Bcl-2 family. New hypothetical model: recent genetic manipulations suggest that mPTP might be an unidentified channel regulated by the proteins constitutive of the classical model. B: outer membrane permeabilization can be due to the formation of pores induced by the activation of Bax by BH-3 only proteins and its translocation to the mitochondrial membrane. It should also be caused by the interaction of Bax with VDAC. It should be noted that VDAC alone could regulate outer membrane permeability and that other mechanisms than Bax and BH-3 only proteins have been involved in outer membrane permeability. However, they were not described during myocardial ischemia-reperfusion.

A. Permeability transition pore



B. Outer membrane permeabilization

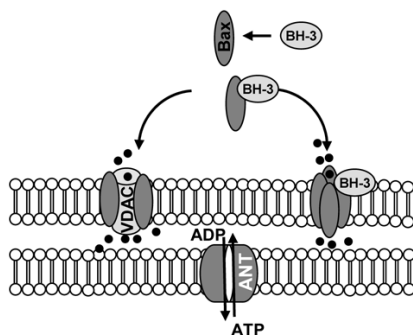


Fig. (3)

The inhibition of mitochondrial permeabilization as a pharmacological approach to limit myocardial ischemia-reperfusion injury

Schematic representation of the pharmacological strategies which have been or might be used to limit mitochondrial membrane permeabilization. They can target directly or indirectly the outer mitochondrial membrane permeability (OMP) or both the outer and the inner membrane (mPTP). CyP-D: cyclophilin D; mPTP: permeability transition pore; VDAC: voltage-dependent anion channel. → : activation ⊥ : inhibition

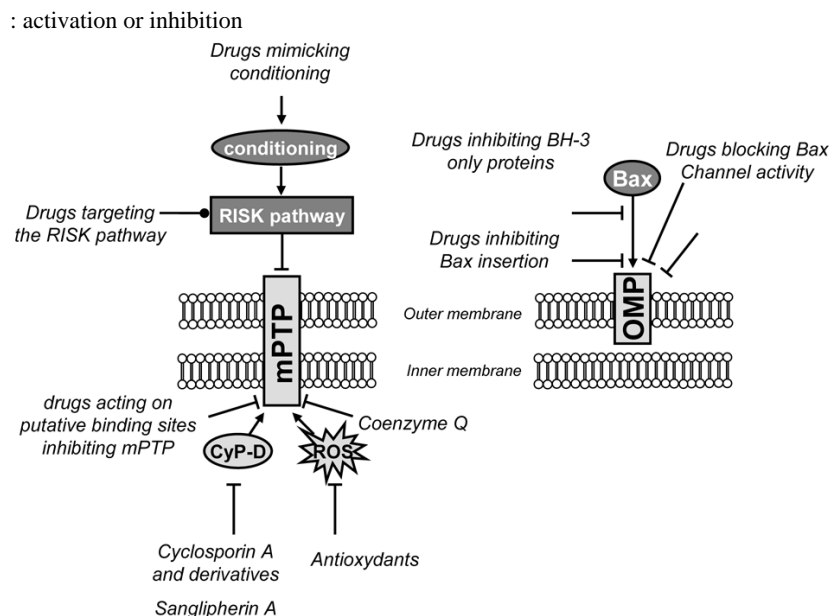
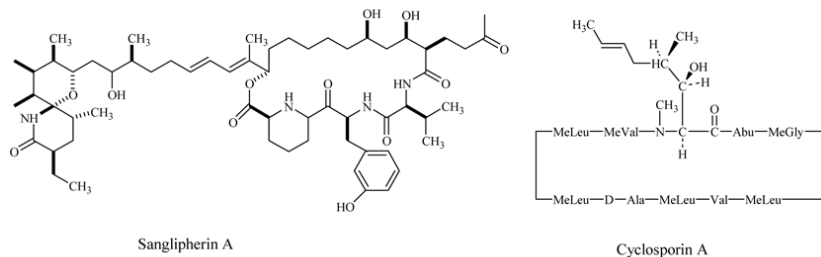


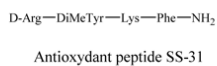
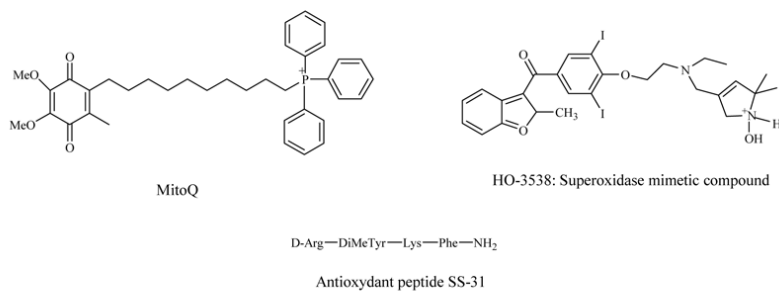
Fig. (4)

Chemical structures of some pharmacological agents capable of limiting mitochondrial membrane permeabilization.

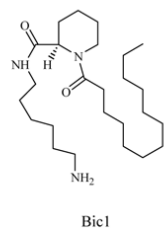
Direct mPTP inhibitors



Mitochondria targeted antioxidants



Bax channel inhibitor



TSPO ligands

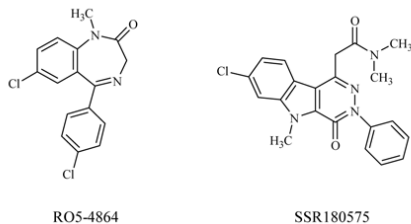


Fig. (5)

Hypothetical scheme showing the cardioprotective pathways activated at the time of reperfusion by both ischemic pre- and post-conditioning. Ligands of receptors coupled to G-proteins or growth factors initiate cardioprotection by activating a cascade of kinase leading to mPTP inhibition and thus protect the cell against apoptotic and necrotic death. mPTP indicates permeability transition pore; K^+_{ATP} : mitochondrial potassium channel. ERK1/2, extracellular regulated kinase; MEK1/2, mitogen-activated protein kinase/ERK1/2 kinase; PI3K, Phosphoinositide 3-kinase; GSK-3 β , glycogen synthase kinase 3 β ; eNOS, endothelial NOS; P70S6K, p70 ribosomal S6 protein kinase \rightarrow : activation \perp : inhibition

