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FORUM REVIEW ARTICLE

Mitochondrial Injury and Protection in Ischemic Pre- and Postconditioning

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Abstract

Mitochondrial damage is a determining factor in causing loss of cardiomyocyte function and viability, yet a mild degree of mitochondrial dysfunction appears to underlie cardioprotection against injury caused by postischemic reperfusion. This review is focused on two major mechanisms of mitochondrial dysfunction, namely, oxidative stress and opening of the mitochondrial permeability transition pore. The formation of reactive oxygen species in mitochondria will be analyzed with regard to factors controlling mitochondrial permeability transition pore opening. Finally, these mitochondrial processes are analyzed with respect to cardioprotection afforded by ischemic pre- and postconditioning. *Antioxid. Redox Signal.* 14, 881–891.

Introduction

Our current understanding of ischemia/reperfusion (I/R) injury is greatly contributed by the notion that mitochondria are both crucial targets of processes triggered by ischemia, such as elevation in intracellular calcium and reactive oxygen species (ROS), and pivotal sites for determining the loss of cell viability. Therefore, it is hardly surprising that strategies aimed at protecting against I/R damage have focused on mitochondria.

The Dual Role of Mitochondrial Dysfunction in Postischemic Reperfusion Injury

Initial evidence of mitochondrial involvement in I/R injury dates back to the 1960s when oxygen consumption was shown to be decreased in mitochondria isolated from dog hearts subjected to 60 min of ischemia (without reperfusion) (60, 61). This *in vitro* finding was followed by the seminal observation that immediately at the onset of reperfusion mitochondria undergo profound morphological alterations (93). In particular, the appearance of dense granules within mitochondrial matrix was recognized to be caused by massive accumulation of Ca^{2+} generating insoluble precipitate of calcium phosphate. Since Ca^{2+} uptake occurs only in mitochondria that utilize oxygen to generate a proton gradient across the inner membrane, this seminal report implies that upon reperfusion mitochondria recover their oxygen consumption to build up a membrane potential that is utilized for Ca^{2+} accumulation within the matrix.

Despite the original cautious comment of Shen and Jennings [whether the calcium uptake is causally related to the development of irreversibility remains to be investigated (3)], mitochondrial calcium overload is likely to play a crucial role in transforming the potentially reversible injury induced by ischemia into the irreversible loss of viability caused by postischemic reperfusion (Fig. 1). In fact, Ca^{2+} uptake antagonizes ATP synthesis since these two processes are driven by the same force, that is, the mitochondrial proton gradient. Not only is ATP synthesis curtailed, but also its hydrolysis is stimulated accelerating ATP depletion. In fact, the increase in matrix Ca^{2+} favors the opening of the mitochondrial permeability transition pore (PTP) (30) that causes a wide array of deleterious consequences, yet it might also be involved in protective mechanisms (49, 50) as discussed in the following sections.

Another relevant concept drawn from those seminal findings is that mitochondrial function is involved in generating the irreversible injury of the heart during reperfusion. This phenomenological association was changed into a causal relationship by Ganote *et al.* demonstrating that the large increase in enzyme release caused by postanoxic reoxygenation is largely prevented by cyanide (41). This paradoxical result provided the concept that cardioprotection, rather than additional injury, is afforded by mitochondrial dysfunction when oxygen supply is reestablished after a prolonged ischemic (or anoxic) episode. This concept has been validated by numerous reports showing that a mild degree of mitochondrial dysfunction confers resistance against I/R injury (24, 38, 40, 67, 71, 86).

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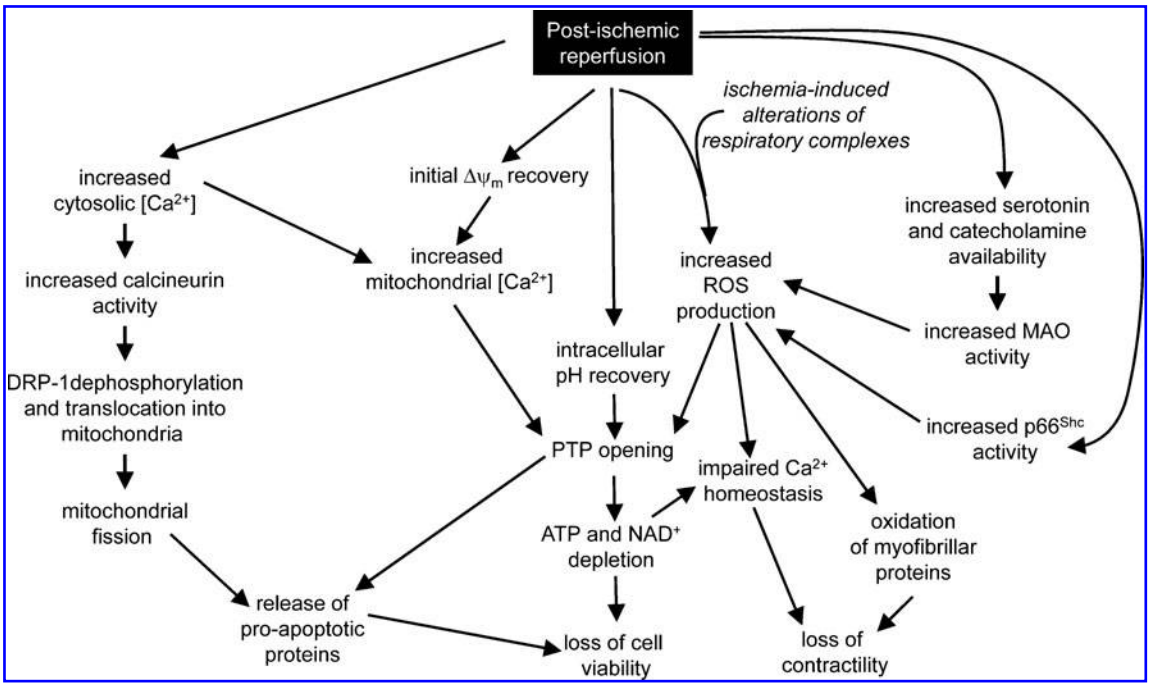

FIG. 1. Major mechanisms linking mitochondrial alterations with cardiomyocyte injury during postischemic reperfusion. MAO, monoamine oxidase; ROS, reactive oxygen species.

During the 1980s, the emphasis on mitochondrial dysfunction was paralleled by increasing attention on metabolic pathways, Ca^{2+} homeostasis, and oxidative stress, yet the interest in mitochondrial processes appeared to move along a sunset boulevard.

During the 1990s the number of mitochondrial studies rose exponentially in almost any biomedical field mostly due to the recognized involvement of mitochondria in apoptosis that from the beginning was linked to PTP (13, 105). Concomitantly, the inhibition of PTP opening by cyclosporine A (CsA) was shown to afford significant cardioprotection against I/R injury (44, 45). At present, a general consensus exists that the PTP is a major factor in determining cell death, and that PTP inhibition affords significant cardioprotection [reviewed in (3, 29, 48)], a concept that has been successfully translated into clinical settings (83).

The PTP in Ischemia/Reperfusion

Major functional and structural changes are induced in mitochondria, and consequently to the whole cell, by PTP opening that causes a sudden increase in the permeability (*i.e.*, permeability transition) of the inner mitochondrial membrane (IMM) to solutes with molecular masses up to 1500 Da (30). The PTP can be defined as a high-conductance channel located in the IMM. Its opening is favored by a decrease in mitochondrial membrane potential, elevation in matrix $[Ca^{2+}]$, and oxidative stress (11).

The protein composition of the PTP has not been identified yet. Evidence from genetic approaches ruled out the possibility that adenine nucleotide translocase and the voltage-dependent anion channel represent PTP components (4, 87). On the other hand, PTP opening is facilitated by binding of the matrix protein cyclophilin D (CyP-D) to the IMM in a process modulated by both Ca^{2+} and inorganic phosphate (Pi) (30). Notably, CyP-D is the target of CsA that reduces the probability of PTP opening by preventing the interaction of CyP-D with the IMM. This effect is mimicked by other compounds that are erroneously described as PTP inhibitors. In fact, since PTP opening can be obtained just by elevating $[Ca^{2+}]$, the effect on the PTP elicited by these compounds should be termed as desensitization. In any case the administration of CsA was an invaluable tool in demonstrating the crucial role of PTP opening in causing the loss of viability in postischemic reperfusion. The initial results obtained with this pharmacological approach combined with biochemical assessments of PTP opening (34, 45) were convincingly corroborated by demonstrating that I/R-induced necrosis is largely reduced in hearts devoid of CyP-D (5, 72).

As far as PTP modulation is concerned, besides the established role of matrix $[Ca^{2+}]$, mitochondrial membrane potential ($\Delta\psi_m$), and ROS, more complex effects are elicited by Pi and H^+ (30). At variance from the established notion that Pi acts a PTP inducer, recent and surprising findings provided evidence that Pi is a PTP inhibitor. In fact, in the absence of this anion the desensitizing effect of CsA or CyP-D ablation is no longer detectable (7). Regarding protons, the accepted concept by which the open probability of the PTP is sharply reduced below pH 7.4 applies only to de-energized mitochondria (74), that is, conditions occurring during prolonged ischemia. Upon reperfusion the rapid recovery of mitochondrial membrane potential might create quite drastic changes. In respiring mitochondria a drop in pH stimulates Pi uptake raising its intramitochondrial content that acts as a powerful PTP inducer. In fact, in energized brain mitochondria acidosis has been reported to promote PTP opening (65).

Although physiological roles of both the PTP and CyP-D have not yet been defined (87), these mitochondrial components are not likely to be involved only in pathological processes. Recent findings obtained in rat liver mitochondria show that CyP-D binds to F_oF_1 ATP synthase, decreasing the rate of ATP hydrolysis (42). It is tempting to speculate that, while protecting from acute injury, CyP-D ablation might compromise the mitochondrial response to conditions of chronic stress. PTP can undergo transient or long-lasting opening depending on the complex balance between cellular inducers and antagonists (82). Opening of short duration is likely to generate reversible cellular changes, so that this modality has been suggested to be involved in physiological processes, such as rapid discharge of excessive intramitochondrial Ca^{2+} (12, 57), intracellular nicotinamide adenine dinucleotide (NAD^+) traffic (35), and transient formation of ROS (101). On the other hand, a prolonged opening of the PTP is followed by profound alterations of cellular bioenergetics that are hardly reversible. Indeed, as detailed in several comprehensive reviews [see, for instance, (11, 29, 46, 107)], the collapse of mitochondrial membrane potential, which is the first and inevitable consequence of PTP opening, is rapidly followed by ATP and NAD^+ depletion, mitochondrial release of accumulated Ca^{2+}, matrix swelling, and rupture of the outer mitochondrial membrane (OMM) leading to the release of proteins, such as cytochrome c, that contribute to determining apoptosis (Fig. 2). Despite mitochondrial deenergization and a modest rise in the levels of Pi, Ca^{2+}, and ROS, during ischemia PTP opening is antagonized by acidosis. Conversely, upon reperfusion and despite an initial recovery of $\Delta\psi_m$ (31), PTP opening is strongly facilitated by pH returning to physiological values along with a large increase in matrix [Ca^{2+}] and ROS accumulation (29) (Fig. 3). The promoting effect of Ca^{2+} is also due to indirect effects that add to its direct action on mitochondria. For instance, arachidonic acid released from phospholipids through the action of Ca^{2+}-dependent phospholipase A_2 (80, 92) and the activation of the Ca^{2+}-dependent proteinase calpain (94) are likely to further facilitate PTP opening (Fig. 4).

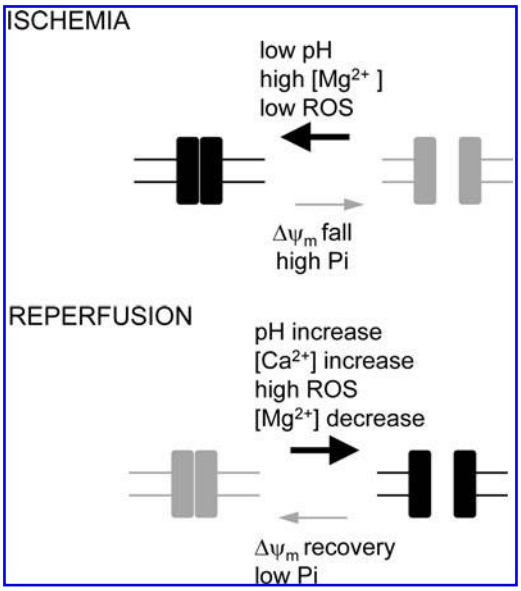

FIG. 3. Factors controlling PTP open probability during ischemia and reperfusion. The equilibrium is likely to be shifted toward the closed state mostly by ischemia-induced intracellular acidosis, whereas upon reperfusion the recovery of pH toward neutral values facilitates the concomitant elevation of matrix [Ca^{2+}] and ROS formation in promoting PTP opening.

Mitochondria and Generation of Reactive Oxygen Species

Although ROS are produced by several intracellular and extracellular processes, in cardiac myocytes mitochondria represent the most relevant site for ROS formation (6, 36, 43, 70, 99). Indeed, even under physiological conditions a minor fraction of oxygen (≤0.1%) undergoes partial reduction to superoxide anion ($O_2^-\cdot$) at the level of Complex I and III rather than being fully reduced to H_2O at the level of Complex

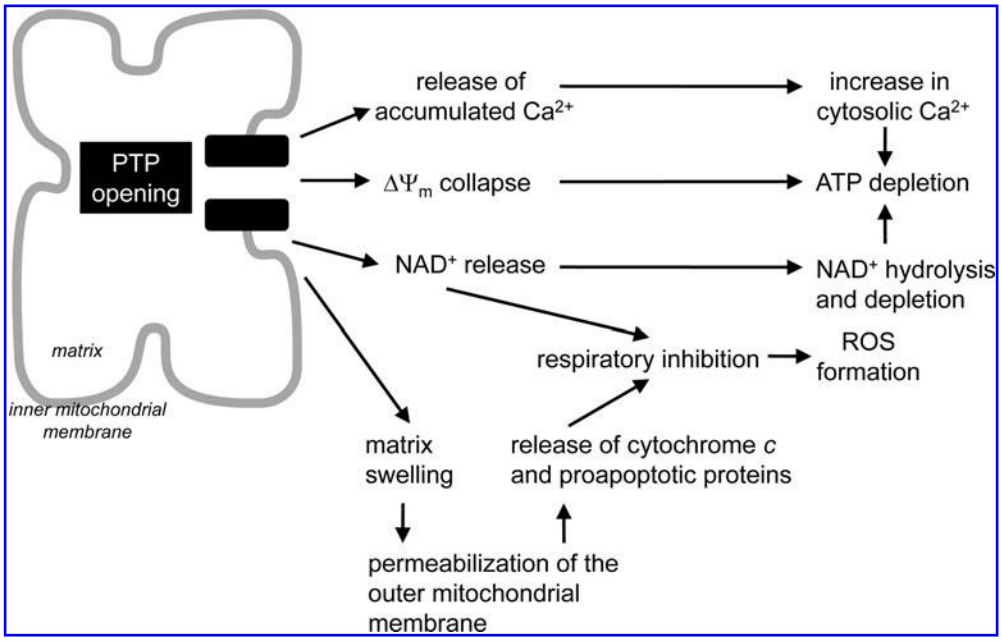

FIG. 2. Major consequences of PTP opening. $\Delta\psi_m$, mitochondrial membrane potential; PTP, mitochondrial permeability transition pore; NAD^+, nicotinamide adenine dinucleotide.

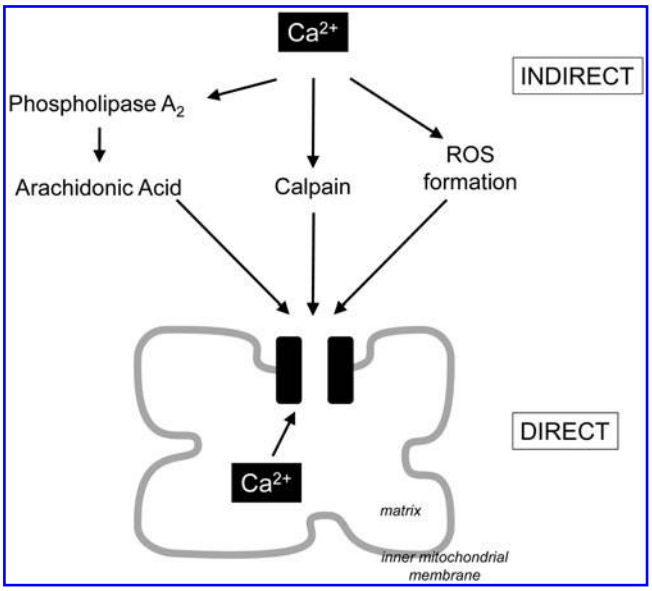

FIG. 4. Relationship between Ca^{2+} and the PTP. A direct action that is usually observed in isolated mitochondria requires nonphysiological levels of Ca^{2+} (*i.e.*, >0.1 mM). Therefore, it is likely that indirect pathways are triggered in response to intracellular [Ca^{2+}] elevations, resulting in the formation of metabolites or stimulating enzyme activities that might sensitize the PTP to Ca^{2+}.

IV. Then, superoxide dismutase (SOD) (39) rapidly reduces $O_2^{-}\cdot$ into H_2O_2 that is freely diffusible through cellular membranes. The further reduction of H_2O_2 to hydroxyl radical (OH·) catalyzed by transition metals, such as Fe^{2+}, represents the most dangerous step. In fact no enzyme is available for the removal of OH· that can only be chemically scavenged by antioxidants with the formation of longer lasting (*i.e.*, less dangerous) radical species.

The mitochondrial formation of ROS might be modulated by NO· (84, 91) as a consequence of the inhibition of cytochrome oxidase (10, 17, 26, 54, 99). This reversible process can be transformed into irreversible alterations of respiratory chain when NO· formation is sustained. Indeed, NO· reacting with $O_2^{-}\cdot$ generates peroxynitrite, which can produce the irreversible nitration of proteins (9).

ROS are also produced within mitochondria at sites other than the IMM (28, 36), such as monoamine oxidase (MAO) and $p66^{Shc}$ (Fig. 5). These additional mitochondrial processes produce significant amounts of ROS. For instance, in brain mitochondria MAO activity results in steady state concentrations of H_2O_2 48-fold higher than those originating from respiratory chain in the presence of antimycin A (20). The relevance of these ROS-generating processes in mitochondria is convincingly supported by several reports showing that MAO inhibition or genetic ablation of $p66^{Shc}$ decrease oxidative stress affording a remarkable degree of protection against I/R injury (14, 22). Beneficial effects of MAO inhibition and $p66^{Shc}$ deletion have also been described in models of vascular injury (21, 73) and heart failure (63).

Besides being a relevant site for ROS formation, mitochondrial function and structure are profoundly altered by oxidative stress (33), especially through a tight relationship with the PTP that has been described as ROS-induced ROS release (106). In fact, ROS facilitate PTP opening (33, 48) that in its turn favors ROS formation by inhibiting electron flow through the respiratory chain due to PTP-induced loss of pyridine nucleotides and cytochrome c. This vicious cycle of injury amplification is likely to be established, especially at the onset of reperfusion when a large increase in ROS formation occurs along with a rise in intracellular [Ca^{2+}] as discussed in the following sections. Besides the severe oxidative stress a wide body of evidence exists demonstrating that a significant formation of ROS occurs during ischemia [(8, 25, 37, 64, 89), and for a review see (97)]. In fact, the inhibition of respiratory chain caused by the insufficient oxygenation facilitates the escape of electrons that can react directly with the available, though scarce oxygen resulting in ROS formation. In fact, mitochondrial ROS formation is favored by a decrease in electron flow resulting from respiratory chain inhibition and is counteracted by uncoupling that is generally produced by an increased IMM permeability to protons. This latter process

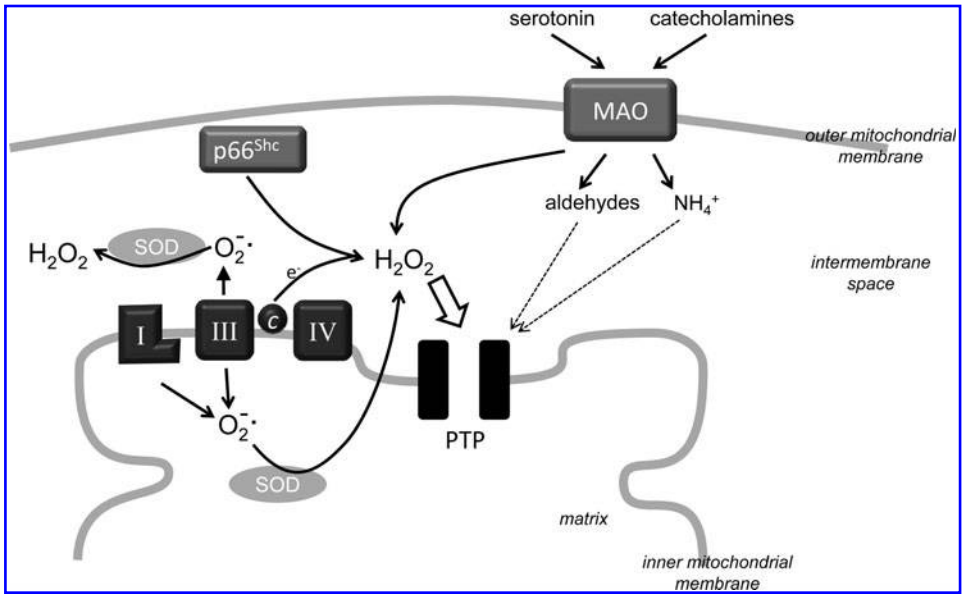

FIG. 5. Mitochondrial sites for ROS formation. Although all these processes appear to be stimulated during myocardial ischemia and reperfusion, the possible mechanisms linking their activities have not yet been elucidated.

explains the protective efficacy related to increased expression of uncoupling proteins (UCPs), especially UCP2 and 3 (90). The level of these proteins is suggested to remain below that required for reducing $\Delta\psi_m$ and ATP synthesis, so that the most relevant effect would remain a decrease in ROS formation. However, despite the large body of evidence supporting the cardioprotective role of UCPs [reviewed in (90)], negative results have also been associated with UCP2 overexpression in cardiomyocytes (15).

PTP and Mitochondrial ROS in Ischemic Pre- and Postconditioning

PTP opening and oxidative stress are not only relevant targets of pharmacological interventions, but also they are involved in endogenous self-defense mechanisms aimed at maintaining cell viability. This is the case with both ischemic preconditioning (IPC) and postconditioning (IPoC) (2, 47, 52, 53, 59, 76) as also discussed in other review articles of this same issue.

A role for the PTP was initially suggested by demonstrating that during reperfusion the extent of PTP opening is reduced in IPC-treated hearts (59, 62). Additional, though more indirect support has been provided by showing that (a) cardioprotection by PTP desensitization is quantitatively similar to that afforded by IPC and IPoC (1, 53), and (b) a decrease in PTP sensitivity to Ca^{2+} is observed in mitochondria isolated at the end of reperfusion from IPC and IPoC-treated hearts (2).

Several mechanisms, not mutually exclusive, have been proposed to explain how IPC and IPoC reduce the susceptibility to PTP opening (Fig. 6). As described in relevant research articles and reviews [for instance, see (56, 69, 76, 103, 107)] IPC and IPoC induce changes in signaling pathways that eventually render mitochondria more resistant to I/R injury. A comprehensive scheme integrating numerous cardioprotective agents has been proposed whereby IPC-induced inactivation of glycogen synthase kinase-3β is transduced to mitochondria, resulting in PTP inhibition (62). Although the evidence was obtained in tumor cells lines, CyP-D is phosphorylated by glycogen synthase kinase-3 increasing the probability of PTP opening (88). While providing a relevant link between signaling pathways and mitochondrial (dys)function, this report further highlights the necessity to solve a major issue in the field. In fact, at present it is not at all clear how processes occurring in the IMM, or even in the matrix as in the case of CyP-D, can be targeted and modulated by cytosolic protein kinases. It is tempting to suggest the effect on energy-linked processes of the IMM results from (de)phosphorylation of critical proteins in the OMM. This possibility is exemplified by the voltage-dependent anion channel, a protein of the OMM. Its phosphorylation has been reported to affect adenine nucleotide traffic between mitochondria and cytosol and/or the binding of the antiapoptotc protein Bcl-2 (96).

Alternatively, or in addition, IMM function might be affected by translocation of cytosolic proteins into mitochondria. This possibility has been suggested for connexin 43 (Cx43) (16, 55), protein kinase Cε (PKCε) (27), and STAT3 (102). For instance, IPC-induced activation of PKCε has been proposed to cause opening of the mitochondrial K_{ATP} channel (mitoK_{ATP}), resulting in a slight increase in H_2O_2 formation, which eventually causes PTP inhibition (27). The ROS-mediated link between PKCε and mitoK_{ATP} might involve additional factors. For instance, the small increase in ROS formation induced by diazoxide is blunted in cardiomyocytes and hearts of heterozygous $Cx43^{+/-}$ mice (55). Therefore, it is tempting to speculate that Cx43 favours PKCε interaction with mitoK_{ATP} or modulates directly mitoK_{ATP} activity.

Not only PTP might be inhibited by a limited oxidative stress, but also a transient or short duration opening of the

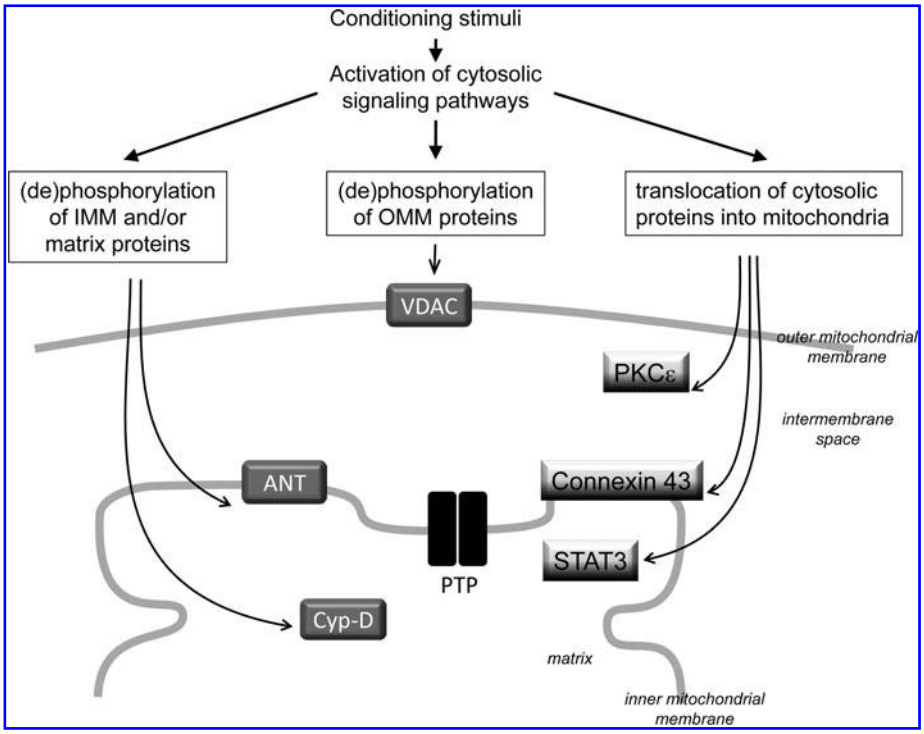

FIG. 6. Possible mechanisms for preconditioning- and postconditioning-induced PTP desensitization. Signaling pathways activated in the cytosol in response to ischemic preconditioning or postconditioning stimuli might reduce the susceptibility to PTP opening during postischemic reperfusion. ANT, adenine nucleotide translocator; IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; PKC, protein kinase C; VDAC, voltage-dependent anion channel.

PTP, a concept introduced by our laboratory (81, 82), has been suggested to induce a minor formation of ROS that might be relevant for cardioprotection. Supporting this concept, genetic and pharmacological inhibition of CyP-D was reported to abrogate both IPC-induced ROS formation and protection (49, 50). ROS might act directly on PTP components or activate signaling pathways that have been suggested to act on mitochondria decreasing their susceptibility to PTP opening. While the modalities of mitochondrial control by cytosolic kinases are still controversial, great attention has been focused on the reperfusion injury salvage kinases pathway (51), which involves the kinases Akt and Erk1/2, and more recently on the survivor activating factor enhancement pathway that has been suggested to contribute to IPoC protection through the activation of tumour necrosis factor-α, its receptor type 2, and STAT-3 (66).

As illustrated in Figure 7, the possibility that PTP opening might be prevented in response to signaling pathways triggered by IPC or IPoC has been argued based upon the observation that susceptibility to PTP opening is not modified (*i.e.*, decreased) in mitochondria isolated at the end of the preconditioning phase, at variance from what is observed at the onset of reperfusion (47). Therefore, changes occuring during ischemia rather than those produced during preconditioning would be responsible for the IPC-induced resistance to PTP opening. This criticism is also accompanied by the observation that in some experimental models activation of the reperfusion injury salvage kinase pathway appears independent of protective effects (25, 95). Rather than causing direct and immediate modifications in protein (de)phosphorylation, IPC reduces the extent of oxidative stress during ischemia and reperfusion (48, 100). Although the evidence is clear that the burst in ROS formation occurring during reperfusion is reduced in IPC-treated cardiomyocytes (100), the mechanism by which IPC or IPoC might affect oxidative stress is far from being elucidated. Since ROS formation and PTP opening are linked in a vicious cycle, at present it can hardly be established whether a reduced oxidative stress is upstream or downstream of a decrease in PTP opening. In addition, evidence that ischemia-induced protein carbonylation is reduced in IPC-treated hearts (48) does not help in clarifying the protective mechanisms. In fact, the carbonylated proteins in mitochondria were not identified and in reported evidence (48) a major contribution might be due to contaminating actin. Moreover, oxidation of mitochondrial proteins is not necessarily linked to adverse effects. For instance, oxidative modification (*i.e.*, nitrosylation) of Complex I has been suggested to be part of the protective mechanisms elicited by IPC (19).

In the context of IPoC, oxidative stress appears to be upstream of PTP opening (Fig. 8). In fact, according to a recent study in rabbit heart undergoing coronary ligation *in situ*, the increase in protein carbonylation induced by postischemic reperfusion was largely decreased by IPoC, but not by CsA (77). Therefore, in the case of IpoC, PTP opening is likely to represent a consequence of ROS accumulation that is required to determine or exacerbate the loss of viability.

Another issue that needs to be addressed is where in mitochondria ROS are generated in IPC and IPoC. Although a general consensus exists that respiratory chain is involved, the possible contribution of other mitochondrial components, namely, MAO and $p66^{Shc}$, has not been investigated. In this respect, $p66^{Shc}$ has recently been reported to be required for IPC in isolated neuronal cells (18).

In IPoC the reduced susceptibility to PTP opening might occur independently of signaling pathways. In fact, postconditioning procedures delay the recovery of intracellular pH that might prevent PTP opening directly and indirectly by inhibiting calpain activation (58). In addition PTP opening might be further prevented by a slight oxidative stress that appears to depend on acidosis (79) and by IPoC-induced decrease in intracellular Ca^{2+} (98). However, a net separation between receptor-dependent and independent processes is not likely to take place in cardiomyocytes during postischemic reperfusion. In fact, (a) not only proteins involved in intracellular Ca^{2+} homeostasis are finely tuned by (de)phosphorylation reactions, but also Ca^{2+} itself is a second messenger. The paramount example of this concept is provided by

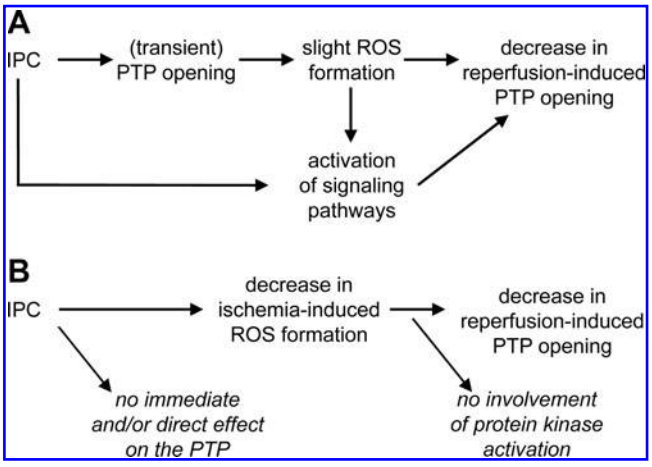

FIG. 7. Mechanisms proposed for IPC-induced decrease in susceptibility to PTP opening during reperfusion. As discussed in the text, the hypothesis **(A)** is mostly related to reports from the laboratory of D. Hausenloy and D. Yellon, whereas the hypothesis **(B)** is supported by findings obtained in A. Halestrap's laboratory. IPC, ischemic preconditioning.

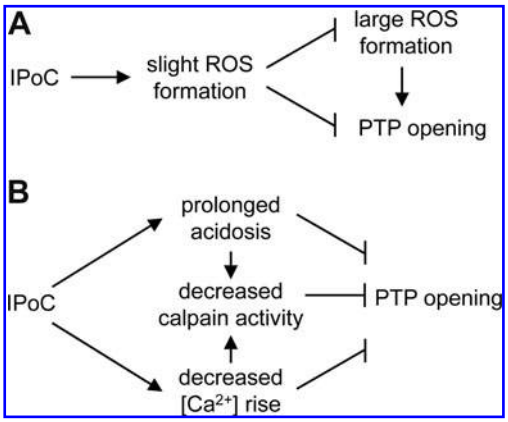

FIG. 8. Mechanisms proposed for IPoC-induced decrease in susceptibility to PTP opening during reperfusion. Notably, the pathways illustrated in the two boxes are not mutually exclusive. **(A)** refers mainly to reports from P. Pagliaro's laboratory, whereas **(B)** summarizes concepts stemming from studies performed in the laboratories of D. Garcia Dorado and J. Vinten-Johansen. IPoC, ischemic postconditioning.

the activation of calcineurin in myocardial hypertrophy (68), and calcineurin has been shown to dephosphorylate dynamin-related protein 1 promoting mitochondrial fission and, consequently, cell death (23). The relevance of this process in cardiac diseases has been highlighted by recent reports (75, 78, 104). (b) Proteases are controlled by covalent modifications. This is also the case with calpain, since the inhibitory activity of calpastatin is modulated by phosphorylation (85). For instance, troponin cleavage by calpain is promoted by PKC phosphorylation and inhibited by protein kinase A phosphorylation of these myofibrillar proteins (32). Similarly, the degradation of other calpain substrates could be modulated by signaling pathways. (c) Mitochondrial dysfunction or protection in response to receptor-dependent stimuli is likely to affect the degree of intracellular acidosis and oxidative stress.

In conclusion, studies aimed at clarifying the mechanisms through which mitochondria affect I/R injury keep attracting a large interest due to the possibility that controlling these processes might provide novel and efficacious therapeutic strategies. The validity of this approach has already been tested successfully in clinical settings (83). The combination of the wide array of experimental protocols and methodological tools that are currently utilized in the field of myocardial I/R injury and protection should allow addressing rapidly the following issues (a) identifying the molecular nature of the PTP, (b) elucidating the mechanisms linking OMM processes with IMM function and structure, and (c) understanding which are the most relevant sources of oxidative stress along with clarifying the conditions and the threshold at which potentially protective ROS become killers out of control.

Acknowledgments

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Abbreviations Used

$\Delta\psi_m$ = mitochondrial membrane potential
CsA = cyclosporin A
Cx43 = connexin 43
CyP-D = cyclophilin D
IMM = inner mitochondrial membrane
IPC = ischemic preconditioning
IPoC = ischemic postconditioning
I/R = ischemia/reperfusion
MAO = monoamine oxidase
mitoK$_{ATP}$ = mitochondrial K$_{ATP}$ channel
NAD$^+$ = nicotinamide adenine dinucleotide
Pi = inorganic phosphate
PKC = protein kinase C
PTP = mitochondrial permeability transition pore
ROS = reactive oxygen species
UCPs = uncoupling proteins
VDAC = voltage-dependent anion channel

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