The mPTP and its regulatory proteins: final common targets of signalling pathways for protection against necrosis

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Abstract

The mitochondrial permeability transition pore (mPTP) is a non-selective, large-conductance channel that is closed under physiological conditions. Opening of the mPTP, leading to abolition of mitochondrial functions, is a major mechanism of myocyte necrosis by ischaemia/reperfusion, and direct inhibition of mPTP opening by use of pharmacological or genetic manipulations limits infarct size in vivo. Multiple pro-survival signal pathways commonly target the mPTP and inhibit its opening. Although the molecular structure of the mPTP has not been established, recent studies have characterized roles of each mPTP subunit and functions of several proteins directly interacting with the mPTP. This article briefly describes the understanding of mPTP regulation and interaction of the mPTP with four proteins (hexokinase II, glycogen synthase kinase-3β, signal transducer and activator of transcription 3, and sirtuin 3) that are downstream of signal pathways relevant to protection from ischaemia/reperfusion injury.

Keywords

Mitochondria • Signal transduction • Hexokinase • Glycogen synthase kinase-3β • Mitochondrial permeability transition pore

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1. Introduction

Myocardial necrosis after ischaemia/reperfusion is an outcome of the combination of ischaemic injury and reperfusion injury.1–3 Relative importance of each component of injury differs depending on the duration of ischaemia, level of residual blood flow in ischaemic tissue, and presumably animal species as well. Recently, accumulating evidence indicates that loss of mitochondrial functions by opening of the mitochondrial permeability transition pore (mPTP) is one of major mechanisms of reperfusion-induced cardiomyocyte necrosis.2–5 However, the role of the mPTP in apoptosis is not clear. Opening of the mPTP induces swelling of mitochondria, leading to rupture of the mitochondrial outer membrane (MOM), and rupture of the MOM results in release of cytochrome c into the cytosol, triggering apoptosome formation. However, the mitochondrial apoptosis-induced channel, which is formed by Bax and Bak in response to pro-apoptotic signals, provides a route for cytochrome c release independent of the mPTP. Since ATP required for apoptosome formation is reduced by mPTP opening, contribution of the mPTP to apoptosis might depend on energy status of the cell and other conditions. A more detailed discussion on the role of the mPTP in apoptosis can be found elsewhere.6,7

Multiple, though not all, pro-survival signal pathways activated by G-protein-coupled receptors and cytokine receptors target the mPTP. The feasibility of myocardial salvage from reperfusion injury by modulating the mPTP has been demonstrated in animal studies and a small clinical trial.8–12 However, for translation of mPTP biology to clinical cardioprotection, it is crucial to understand the mechanism by which mPTP opening is regulated and how we can modify the regulation. Since we recently reviewed pro-survival signal pathways targeting mitochondria in the journal (Figure 1), we focus on the interaction of the mPTP subunits and proteins relevant to protection from necrosis after ischaemia/reperfusion in this article. The following discussion is based on data from both cardiac and non-cardiac cells, since data from cardiomyocytes for this issue are relatively limited.

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2. Components of the mPTP complex and their regulation under ischaemia/reperfusion

Molecular structure of the mPTP remains unclear and different models have been proposed. A classic model of the mPTP consists of a dimer of voltage-dependent anion channel (VDAC) in the mitochondrial outer membrane (MOM) and a dimer of adenine nucleotide translocase (ANT) in the mitochondrial inner membrane (MIM) forming a channel. This model is no longer widely supported since genetic ablation of ANT or VDAC did not eliminate mPTP opening, which was detected by mitochondrial swelling and by change in mitochondrial membrane potential ($\Delta \Psi_m$). However, roles of ANT and VDAC as regulatory subunits of the mPTP together with a matrix protein, cyclophilin D (CyPD), have been indicated by several lines of evidence as discussed below. In addition, a recent study by Leung et al. suggests that mitochondrial phosphate carrier [inorganic phosphate carrier (PiC)] is also a major subunit of the mPTP, possibly forming a channel in the MIM. A putative structure of the mPTP is a complex across the mitochondrial membrane, consisting of VDAC in the MOM and ANT–PiC complex in the MIM (Figure 2).

Ischaemia and reperfusion induce metabolic changes that facilitate opening of the mPTP in cardiomyocytes (Figure 3). However, the mPTP remains closed during ischaemia and opens shortly after reperfusion as demonstrated by determination of influx of mPTP remains closed during ischaemia and opens shortly after reperfusion as demonstrated by determination of influx of Ca$^{2+}$, but timing of CyPD–mPTP binding during ischaemia/reperfusion and molecular mechanisms of the binding are not clearly understood. A model of the functional relationships between mPTP regulatory factors is presented in Figure 4, but effects of ischaemia and reperfusion on each of the regulatory factors remain unclear except for CyPD–ANT/PiC interaction upon reperfusion.

2.1 Voltage-dependent anion channel

There are three isoforms of VDAC (VDAC1, VDAC2, and VDAC3) encoded by separate genes. Of the isoforms, VDAC1 is the most abundant in most cells and interacts with multiple proteins involved in energy production, apoptosis, and formation of cytoskeletal frameworks. VDAC in the MOM binds to hexokinase II (HKII), which sets a machinery of ADP transport into mitochondria and a machinery of ADP production (from phosphorylation of glucose by ATP) in a proximate location. The VDAC–HKII interaction can be disrupted either by treatment with TAT-HKII, a cell-permeable HKII N-terminal peptide, or by overexpression of HKII with mutations in N-terminal regions (putative HK-interacting sites). Dissociation of HKII from mitochondria by TAT-HKII induces mitochondrial depolarization due to opening of the mPTP as discussed in a section on HKII below. Phosphorylation of VDAC by glycogen synthase kinase-3β (GSK-3β) also induces release of HKII from the mitochondria at least in non-cardiac cells. Furthermore, enhanced tolerance to oxidant-induced necrosis was afforded by HKII overexpression, which was associated with phosphorylation of VDAC by protein kinase C-ε (PKC-ε). The protection afforded by HK overexpression was eliminated by HKII detachment from VDAC. These findings indicate that VDAC contributes to maintenance of mPTP closure by anchoring HKII, which modifies VDAC–PKC-ε interaction, in mitochondria.

In addition to GSK-3β and PKC-ε, PKA phosphorylates VDAC. PKA-mediated phosphorylation of VDAC suppresses open probability of VDAC, which would reduce transport of ADP to the mitochondrial matrix. Conversely, GSK-3β-mediated phosphorylation of VDAC increases adenine nucleotide transport into the matrix in cardiomyocytes, though supply of ADP derived from HKII might be reduced to some extent due to HKII dissociation by VDAC phosphorylation. Nevertheless, since ATP hydrolysis by mitochondrial ATPase (F1F0 ATPase) for maintenance of $\Delta \Psi_m$ accounts for
35–50% of ATP consumption during ischaemia, the level of VDAC phosphorylation by PKA and GSK-3β is likely to determine rates of ATP depletion and decline of $\Delta \Psi_m$ in ischaemic cardiomyocytes.

### 2.2 Adenine nucleotide translocase

ANT localizes in the MIM and exchanges ADP$_{32}$ and ATP$_{42}$ between the mitochondrial matrix and the intermembrane space. This transporter function is positively regulated by several factors, including the two substrates (ADP and ATP) and Bcl-2. The ANT family consists of four isoforms encoded by different nuclear genes. ANT1 is the predominant isoform in the heart and muscle and ANT3 is expressed at low levels independently of cell types. ANT2 and ANT4 are expressed in proliferative tissues and in the brain, liver, and testis, respectively. Each isoform appears to have distinct roles since the effect of overexpression of ANT on cell viability is opposite depending on the isoform (anti-survival ANT1 and ANT3 vs. pro-survival ANT2 and ANT4).

A regulatory function of ANT in the mPTP has been indicated by several lines of evidence. Opening of the mPTP by oxidant stress was closely associated with cross-linking at Cys160 to Cys257 in ANT. Elevation of matrix Ca$_{2+}$ level, a trigger of mPTP opening, induced the 'c-conformation' (cytosolic-open state) of ANT, and ANT-specific agents, carboxyatractyloside and bongkrekic acid, had positive and negative effects, respectively, on sensitivity of the mPTP to matrix Ca$_{2+}$. Furthermore, Ca$_{2+}$ sensitivity of the mPTP was significantly reduced in mitochondria in which ANT was genetically ablated. Impact of ANT carbonylation on mPTP sensitivity is also supported by the findings that ANT1 was the only protein in the mitochondrial membrane showing an age-associated increase in carbonylation and that susceptibility to mPTP opening is increased by ageing.

Physical interaction of ANT with CyPD and PiC was demonstrated by glutathione S-transferase (GST) pull-down experiments using GST–CyPD fusion proteins. Treatment with cyclosporine A did not affect this CyPD–ANT interaction, but it inhibited binding of CyPD to PiC. The ANT–PiC interaction was not inhibited by cyclosporine A. These findings argue against a previous notion that CyPD binding to ANT increases sensitivity of the mPTP to Ca$_{2+}$. Change in ANT–PiC interaction rather than that in ANT per se might be responsible for changes in Ca$_{2+}$ sensitivity of the mPTP by ANT ablation and by cyclosporine A treatment as discussed in the following section on PiC. However, this possibility has not been critically examined.

### 2.3 Inorganic phosphate carrier

There are two PiC isoforms (PiC-A and PiC-B), which are alternative splicing products from a single PiC gene (SLC25A3). PiC-A is expressed in cardiac and skeletal muscles, and PiC-B is ubiquitously expressed. PiC localizes in the MIM and functions as a Pi–H$^+$ symport and/or Pi–OH$^2$ antiport. Nevertheless, PiC catalyses transport of cytosolic Pi to the mitochondrial matrix, where Pi is used for ATP synthesis. A recent study using INS-1 cells demonstrated that the Pi level as well as the protein level of PiC determines the rate of ATP production in mitochondria. Knockdown of PiC alone did not affect $\Delta \Psi_m$, indicating that reduced ATP production by PiC knockdown is not due to loss of protomotive force.

PiC has been shown to interact with other mPTP subunit proteins. Binding of PiC with CyPD and ANT in rat cardiac and liver mitochondria has been demonstrated by GST pull-down assay using GST–CyPD fusion protein. In 293T cells and HeLa cells, PiC was co-immunoprecipitated with ANT1 and VDAC1. Interestingly, the ANT1–PiC interaction was increased when apoptosis was induced by staurosporine in HeLa cells, and knockdown of PiC suppressed...
cytochrome c release from mitochondria and apoptosis. Response of ANT1–PiC interaction to necrosis-inducing stimuli remains unclear. As mentioned in a previous section, Leung et al.15 found that PiC interacts with CyPD in a cyclosporine A-sensitive manner. Using column-immobilized phenylarsine oxide (PAO), an mPTP-activating agent, they showed that both PiC and ANT bind to PAO and that this binding was inhibitable by N-ethylmaleimide (NEM) and ubiquinone analogues (Ro68-3400, UQ0), mPTP inhibitors which do not inhibit peptidyl-prolyl cis–trans isomerase (PPIase). Interestingly, NEM and ubiquinone analogues suppressed Pi transport into the mitochondria. Although information on mammalian PiC is lacking, PiC of yeast dimerized by a dithiol cross-link has been shown to form a non-specific anion channel.45 Based on these findings, Leung et al.15 proposed a model of mPTP consisting of a channel-forming PiC dimer and a dimer of ANT.

Leung et al.15 showed a correlation between mitochondrial Pi influx via PiC and threshold for opening of the mPTP. However, cyclosporine A did not modify PiC-mediated Pi influx into mitochondria, indicating that inhibition of the mPTP opening by cyclosporine A is not achieved by change in Pi influx per se. In this regard, interesting observations have been reported by Basso et al.46 They showed that inhibition of mPTP opening by cyclosporine A or CyPD knockout disappeared when Pi was replaced by its analogue, arsenate or vanadate. The effect of Pi on threshold for mPTP opening was dose-dependent and unaffected by increasing concentrations of co-existing arsenate or vanadate. Based on these findings, Basso et al.46 proposed that cyclosporine A and CyPD knockout inhibits mPTP opening by unmasking inhibitory effects of Pi.

3. Proteins directly regulating the mPTP in mitochondria

3.1 Cyclophilin D
CyPD is one of the cyclophilin family proteins with PPlase activity and is the only isoform localizing in the mitochondrial matrix.47 Although the physiological function of CyPD in cardiomyocytes was unclear until recently, a study by Elrod et al.48 demonstrated that knockout of the Ppif gene (a gene encoding CyPD) induced elevation of mitochondrial Ca$^{2+}$ level, activation of Ca$^{2+}$-dependent enzymes, and metabolic re-programming, resulting in reduction in ventricular tolerance to pressure overload. Their findings indicate that the physiological function of CyPD is regulation of mitochondrial Ca$^{2+}$ level for metabolism to be matched with mechanical workload.

A significant role of CyPD in cardiomyocyte necrosis after ischemia/reperfusion has been well established. Treatment with cyclosporine A or genetic ablation of CyPD elevates the threshold for opening of the mPTP and consistently affords tolerance against ischemia/reperfusion-induced necrosis.8–12 As discussed in previous sections, regulation of the mPTP by CyPD is achieved by interaction with PiC and ANT, which modifies sensitivities of the mPTP to Ca$^{2+}$ and Pi.

The mitochondrial ATPase is also a target of CyPD. Giorgio et al.49 showed that CyPD interacts with the lateral stalk of the mitochondrial ATPase and reduces its activity. Recently, Chinopoulos et al.50 assessed the impact of CyPD-mediated reduction in the mitochondrial ATPase on ATP efflux via ANT and matrix pool of adenine nucleotides. Their results indicate that ANT-mediated ATP efflux in energized mitochondria or ATP influx in de-energized mitochondria is not significantly changed by CyPD-mediated suppression of mitochondrial ATPase activity because flux control coefficient of mitochondrial ATPase is ~2.2-fold lower than that of ANT. However, two important functions of the mitochondrial ATPase regulation by CyPD were proposed by Chinopoulos et al.50 One is regulation of matrix adenine nucleotide level and the other is inhibition of mPTP opening via accelerated H$^+$ pumping by ATP hydrolysis, leading to more robust maintenance of $\Delta\Psi_m$.

3.2 Hexokinase II
Of the four isoforms of HK, HKI and HKII localize in both the cytosol and mitochondria of cardiomyocytes,51 and their binding to mitochondria is mediated by a hydrophobic N-terminal domain.21–23 HKI and HKII differ in affinities for glucose and ATP,53 allosteric regulation,51 and response of their expression to myocardial infarction.54

Figure 4 A model of relationship between mPTP regulatory factors. Ischaemia/reperfusion has been shown to induce CyPD–PiC/ANT interaction in cardiomyocytes, though its effect on VDAC phosphorylation and HKII binding to VDAC remains unclear. MitoATPase, mitochondrial ATPase. See text for details.
Thus, it is likely that HKI and HKII play different roles in regulation of glucose metabolism. The role of HKI in regulation of mitochondrial functions, if any, remains unclear. On the other hand, contribution of HKII to mPTP regulation has been supported by several lines of evidence.

3.2.1 Regulation of HKII binding to mitochondria
HKII binding to mitochondria is increased by ischaemic preconditioning (IPC), insulin, and morphine.55–58 These treatments activate PI3K-Akt signalling, and an inhibitor of PI3K or genetic ablation of Akt abolished the HKII recruitment to mitochondria. Transfection with constitutively active Akt increased HK activity in mitochondria.55,56 Conversely, phosphorylation of VDAC by GSK-3β induced dissociation of HKII from VDAC in tumour cells, and this process was inhibited by activation of Akt.60 These findings suggest that PI3K-Akt signalling, leading to inactivation of GSK-3β, is a primary mechanism of the promotion of HKII translocation to mitochondria.

HKII release is also induced by inhibition of PPlase using cyclosporine A or clotrimazole in HeLa cells.59,60 Recently, Shulga et al.60 reported that deacetylation of CyPD by sirtuin 3 (SIRT3), which reduces its PPlase activity, induced dissociation of CyPD from ANT1 and also dissociation of HKII from mitochondria in the same cell line. Interestingly, atracyloside, which binds to ANT and fixes it in the c-conformation, was shown to promote HKII binding to VDAC in HeLa cells.61,62 Taken together, the findings indicate that there may be two mechanisms of HKII release from the mitochondria in HeLa cells. One is GSK-3β-mediated phosphorylation of VDAC, a binding partner of HKII, and the other is a conformational change of ANT, which is under regulation of PPlase activity of CyPD. It is not clear whether similar mechanisms are present in cardiomyocytes.

3.2.2 Inhibition of mPTP opening by HKII and its potential mechanisms
Several lines of evidence indicate that mitochondrial HKII suppresses opening of the mPTP. Dissociation of HKII from mitochondria by use of cell-permeable peptide analogues (n-HKII and TAT-HKII) specifically induced mitochondrial depolarization and cell necrosis in both cardiomyocytes and HeLa cells.25,63,64 Cell death (mostly necrosis) induced by TAT-HKII was attenuated by an inhibitor of mPTPs (Debio 025) or by knockdown of CyPD.22 In neonatal rat cardiomyocytes, n-HKII induced mitochondrial depolarization and cell death dose dependently.25 Mitochondrial depolarization by a high dose of TAT-HKII (10 μM) was observed also in intact mouse hearts, in which ΔΨm was monitored by tetramethylrhodamine ethyl ester.64 A lower dose of TAT-HKII (200 nM) enhanced susceptibility to ischaemia/reperfusion-induced necrosis and prevented protection of IPC.61 These results are consistent with the notion that dissociation of HKII from the mPTP complex lowers the threshold for opening of the mPTP.

How HKII detachment from mitochondria facilitates mPTP opening remains unclear. One possibility is augmentation of ROS production. A series of studies using brain and liver mitochondria indicates that HKII associated with VDAC plays a major role in the ADP recycling mechanism in mitochondria, suppressing ROS production and maintaining ΔΨm.65,66 Preservation of ATP production is another potential mechanism by which mitochondrial HKII inhibits mPTP opening. In fact, knockdown of HKII reduced ATP production by ~20% in cardiomyocytes.25 Another possible mechanism is modulation of ANT conformation in the mPTP. In a study by Chiara et al.,72 locking ANT in the ‘m conformation’ (matrix-open state) by bongkrekic acid suppressed cell death induced by TAT-HKII, whereas activation of Akt-GSK-3β signalling using insulin did not affect cell death.

3.3 Glycogen synthase kinase-3β
3.3.1 Regulation of GSK-3β activity and translocation
GSK-3β is one of the two isoforms of GSK-3, a Ser/Thr protein kinase. GSK-3β is constitutively active and its activity is increased by phosphorylation at Tyr216.67 This Tyr is autophosphorylated, though the possibility of involvement of an unidentified protein kinase has not been excluded. Inactivation of GSK-3β is achieved by phosphorylation at Ser9, and multiple protein kinases (such as PKA, PKC, and ERK) target this Ser residue. In addition, Thr43 and Ser389/Thr390 in GSK-3β are phosphorylated by ERK and p38-mitogen-activated protein kinase, respectively, and phosphorylation of these residues is thought to facilitate Ser9 phosphorylation by other kinases.68,70

Since substrates of GSK-3β localize different intracellular compartments, translocation into subcellular compartments (i.e. nucleus and mitochondria) is an important regulatory mechanism of GSK-3β functions. In cardiomyocytes, GSK-3β is predominantly localized in the cytosol under an un-stimulated condition, but ischaemia/reperfusion induces translocation of GSK-3β from the cytosol to mitochondria.71 Although the trigger of translocation remains unclear, our preliminary study suggests that oxidant stress is involved.72 GSK-3β was co-immunoprecipitated with translocase of the mitochondrial outer membrane 20 (TOM20), indicating the possibility that GSK-3β translocation to mitochondria is mediated by the TOM20-heat shock protein 90 mechanism as are translocations of connexin-4373 and PKC-ε.74

Multiple cytoprotective signalling pathways converge on GSK-3β to induce its phosphorylation at Ser9,17 (Figure 1). Cardioprotection afforded by activation of the erythropoietin (EPO) receptor or the mitochondrial ATP-sensitive K⁺ channel (mKATP channel) before ischaemia was associated with an increase in mitochondrial Ser9-phospho-GSK-3β upon reperfusion without change in total GSK-3β level in mitochondria.75,76 To address the relationship between timing of GSK-3β phosphorylation and cardiomyocyte protection, we recently examined the effects of activation of the mKATP channel on GSK-3β phosphorylation before and after ischaemia/reperfusion in rat hearts.76 We focused on the mKATP channel since its activation is a common step downstream of activated receptors during the trigger phase of IPC and its mimetics.3,17

In contrast to our expectation, activation of the mKATP channel by diazoxide failed to induce phosphorylation of GSK-3β in the normoxic myocardium, though ROS produced by mKATP channel opening should have induced activation of PKC-ε, an upstream kinase of GSK-3β. During ischaemia, GSK-3β undergoes dephosphorylation, though this ischaemia-induced dephosphorylation is not specific to this kinase and is observed in Akt, ERK1/2, and p70S6 kinase.77,78 On the other hand, mitochondrial level of phospho-GSK-3β upon reperfusion was significantly elevated in diazoxide-pre-treated hearts.79 These results indicate that intracellular signalling activated by the mKATP channel before ischaemia enhances GSK-3β phosphorylation in mitochondria upon reperfusion.

As a mechanism of the link between pro-survival signalling before ischaemia and that at the time of reperfusion in IPC, Downey and his colleagues have proposed that PKC-ε activated by mKATP channel opening sensitizes the low-affinity adenosine A2b
3.3.2 Inhibition of mPTP opening by phosphorylation of GSK-3β

Inhibition of mPTP opening is one of mechanisms that have been proposed for cytoprotection afforded by phosphorylation of GSK-3β at Ser9.93–94 Two distinct mechanisms may be involved in the mPTP inhibition by phosphorylation of GSK-3β: inactivation of its kinase activity and interaction of phospho-GSK-3β with mPTP regulatory proteins (Figure 4). Phosphorylation of GSK-3β at Ser9 and knockdown of GSK-3β have been shown to inhibit opening of the mPTP in response to ROS or Ca2+ overload in cardiomyocytes. Inactivation of GSK-3β can decrease phosphorylation of VDAC, which potentially suppresses HKII release from mitochondria and also nucleotide influx into the matrix. Prevention of such HKII release from mitochondrial HKII may inhibit mPTP opening as discussed above, and reduced flux of adenine nucleotides into the matrix during ischemia theoretically slows depletion of ATP, a determinant of mPTP sensitivity.

Phosphorylation of GSK-3β at Ser9 modifies its interaction with mPTP subunits. In our series of experiments, GSK-3β was co-immunoprecipitated with ANT and VDAC, which was increased after ischemia/reperfusion.71 In contrast, Ser9-phospho-GSK-3β interacted with ANT but not with VDAC, and this phospho-GSK-3β–ANT interaction was associated with reduction in CyPD–ANT interaction. Furthermore, activation of P13K–Akt–GSK-3β signalling before ischemia, an increase in phospho-Ser9-GSK-3β–ANT interaction, and a decrease in CyPD–ANT interaction upon reperfusion were commonly observed for protection afforded by IPC,71 activation of EPO receptor (data showing EPO-induced suppression of CyPD–ANT complex formation are unpublished),76 and activation of the mKATP channel.76 Changes in the CyPD–ANT interaction by GSK-3β phosphorylation might reflect changes in CyPD–PIC interaction, since GSK-3β phosphorylation has the same impact on the mPTP as cyclosporine A treatment11,17 and the ubiquinone analogues (Ro68-3400, UQO) that inhibit PI transport in mitochondria induce conformational changes of ANT.15 However, this possibility remains to be directly examined.

3.4 Signal transducer and activator of transcription 3

3.4.1 Functions of signal transducer and activator of transcription 3 in mitochondria

Signal transducer and activator of transcription 3 (STAT3) is one of the seven STAT isoforms expressed in the heart.86 As in the case of other isoforms, STAT3 is phosphorylated by activated Janus kinase (JAK), and the phosphorylated STAT3 binds to promoter regions of target genes, modulating their transcriptions. STAT3 plays a distinct role in mitochondria.87–89 STAT3 localizes in the matrix of cardiac mitochondria, being dually phosphorylated at Tyr705 and Ser9.83,84 Two distinct mechanisms may be involved in the mPTP inhibition by phosphorylation of STAT3: inactivation of its kinase activity and interaction of phospho-STAT3 with mPTP regulatory proteins (Figure 4). Phosphorylation of STAT3 at Tyr705 and knockdown of STAT3 have been shown to inhibit opening of the mPTP in response to ROS or Ca2+ overload in cardiomyocytes. Inactivation of STAT3 can decrease phosphorylation of VDAC, which potentially suppresses HKII release from mitochondria and also nucleotide influx into the matrix. Prevention of such HKII release from mitochondrial HKII may inhibit mPTP opening as discussed above, and reduced flux of adenine nucleotides into the matrix during ischemia theoretically slows depletion of ATP, a determinant of mPTP sensitivity.

Phosphorylation of STAT3 at the time of reperfusion by use of IPost, leptin, or TNF-α successfully limited infarct size after ischemia/reperfusion.71 However, an increase in Ca2+ retention capacity of mitochondria by cyclosporine A was unaffected by STAT3 ablation or by Stattic,88 indicating that the inhibitory effect of STAT3 on the mPTP is not mediated by modulation of PPIase activity of CyPD.

3.4.2 STAT3 in protection against ischemia/reperfusion-induced necrosis

A role of STAT3 in myocardial protection from necrosis has been demonstrated for IPC,90 ischaemic postconditioning (IPost),91 and ligands of JAK-coupled receptors (TNF-α, insulin, and leptin).91–94 All of these cardioprotective interventions induce phosphorylation of STAT3 at Tyr705, and the protective effects of the interventions were lost by genetic ablation of STAT3 or an inhibitor of JAK (AG-490). Whether ‘mitochondrial’ STAT3 is responsible for the protection afforded by IPC, IPost, and the receptor ligands has not been critically examined. However, it is unlikely that the role of STAT3 as a transcriptional factor is mainly involved in the protection since activation of STAT3 at the time of reperfusion by use of IPost, leptin, or TNF-α successfully limited infarct size after ischemia/reperfusion.

3.5 Sirtuin 3

3.5.1 Functions of SIRT3 in mitochondria

Emerging evidence suggests that Lys residue acetylation is an important post-translational modification to regulate various biological functions, including the function of the mitochondria.95 Sirtuins mediate this post-translational modification by coupling lysine deacetylation to NAD+ hydrolysis.96 Mammals express seven sirtuins, and three of them (SIRT3, SIRT4, and SIRT5) localize in mitochondria.97–99 SIRT3 has received attention for its role in cell survival under stress. The full-length human SIRT3 is a 44 kDa protein with an N-terminal mitochondrial localization sequence.100 Following import into mitochondria, 142 amino acids from the N-terminal of full-length SIRT3 are cleaved to generate an active 28 kDa short form.

Included among the target proteins for SIRT3 are acetyl-CoA synthetase 2 (AceCS2) and long-chain acyl-CoA dehydrogenase (LCAD). AceCS2 promotes entry of acetyl into the tricarboxylic acid cycle in the form of acetyl-CoA, where it can be used for ATP production. SIRT3 interacts with AceCS2 in mitochondria, where SIRT3 deacetylates Lys642 of the protein and activates its enzymatic activity.101 LCAD is hyperacetylated at Lys42, resulting in reduction in its activity, in the absence of SIRT3.102 These findings suggest a role of SIRT3 in LCAD-mediated control of fatty acid oxidation output. It has also been shown that SIRT3 physically interacts with NDUFS9, a subunit of complex I of mitochondrial electron transport chain, deacetylates and activates the enzyme, and thus augments ATP production.102 Taken together, the findings suggest that SIRT3 regulates ATP production at multiple steps. In fact, mice lacking SIRT3, which exhibit a striking hyperacetylation of mitochondrial proteins,98 showed 50% lower basal levels of ATP in the heart.104

3.5.2 SIRT3 and the mPTP

The role of SIRT3 in regulation of the mPTP is still largely unknown, but interesting observations have recently been reported in this regard. Shulga et al.88 showed that SIRT3 deacetylates CyPD and
inhibits its PP1ase activity in HeLa cells, and Hafer et al. confirmed the CyPD deacetylation at Lys166 by SIRT3 in cardiac tissue. Furthermore, they demonstrated that an increase in Ca\(^{2+}\) sensitivity of the mPTP by ageing was significantly enhanced in cardiac mitochondria isolated from SIRT3-knockout mice. This finding indicates that SIRT3 counteracts an age-induced increase in sensitivity of the mPTP, which might be partly, at least, due to loss of mitochondrial SIRT3. Although SIRT3-mediated inhibition of CyPD activity is a possible explanation for the protection afforded by SIRT3 in aged animals, the impact of SIRT3 deletion on CyPD activity has not been directly examined. Furthermore, interaction of SIRT3 with mPTP subunits besides CyPD and indirect effect of SIRT3 on the mPTP via mitochondrial metabolism also remain unclear.

4. Dispute over mPTP regulation by mitochondrial kinases

Results of a study by Clarke et al. argue against a role of mitochondrial kinases in inhibition of mPTP. They prepared pure mitochondrial fractions by using Percoll gradient and determined phosphorylation of proteins by one- or two-dimensional electrophoresis and anti-phosphoprotein antibodies or Pro-Q Diamond. Their thorough analyses, which were performed with few cardioprotective agents, indicated that neither phosphorylation nor recruitment of PKC-ε, Akt, or GSK-3β to mitochondria occurs after IPC or after reperfusion with or without IPC. The difference between the results of the study by Clarke et al. and results of other studies indicating IPC-induced phosphorylation/rerecruitment of mitochondrial kinases might be explained by different levels of contamination of non-mitochondrial membranes in mitochondrial fractions.

However, another possible explanation is isolation of different groups of mitochondria. The use of Percoll gradient certainly improves purity of mitochondrial fractions from the normal myocardium, but it is not clear whether the same method is suitable for isolation of mitochondria from the myocardium subjected to ischaemia/reperfusion. The myocardium after ischaemia/reperfusion consists of viable and necrotic cells, and severity of mitochondrial injury should be heterogeneous within the tissue. Hence, different groups of mitochondria could have been unintentionally selected depending on methods of their isolation. This possibility is consistent with the difference in apparent change in Ca\(^{2+}\) sensitivity of the mPTP by IPC between the study by Clarke et al. and studies by others. Ischaemia/reperfusion increases Ca\(^{2+}\) sensitivity of the mPTP and facilitates its opening in cardiomyocytes. Surprisingly, in the study by Clarke et al., mPTPs in preconditioned hearts after ischaemia/reperfusion were less Ca\(^{2+}\)-sensitive even compared with mPTPs in control hearts before ischaemia. However, such desensitization of the mPTP in the reperfused myocardium beyond the normal control level was not observed in studies by Ovize and his colleagues, though IPC attenuated an increase in Ca\(^{2+}\) sensitivity of the mPTP after ischaemia/reperfusion.

5. Modification of the mPTP by concurrent disease

Recent studies using animal models of heart failure, diabetes, and hypertension have demonstrated that the threshold for mPTP opening is significantly reduced by pathological conditions. Increased ROS production appears to be a common mechanism, but it is notable that changes in the mPTP component differ between the models. Mitochondrial CyPD was up-regulated and the mPTP was hypersensitive to Ca\(^{2+}\) in a model of volume overload-induced heart failure. We found that active (i.e. non-phosphorylated) GSK-3β was increased in mitochondria in type 2 diabetes, and the up-regulation of mitochondrial GSK-3β was correlated with increased sensitivity of the mPTP to Ca\(^{2+}\). A recent human study confirmed that sensitivity of the mPTP to Ca\(^{2+}\) was indeed higher in mitochondria from atria of diabetic patients than that in non-diabetic controls. In a model of hypertension, protein levels of the mPTP subunit proteins were comparable, though carbonylation of mitochondrial proteins after ischaemia/reperfusion was significantly enhanced. Hence, repair of mPTP dysregulation appears to be as important as activation of mPTP inhibitory mechanisms in the strategy for protecting the heart from mPTP-mediated cell necrosis.

6. Conclusion

Roles of VDAC, ANT, and PC, as subunits in the mPTP complex, have been supported by results of studies to date. Included among proteins directly regulating open probability of the mPTP are CyPD, HKII, GSK-3β, STAT3, and SIRT3. Binding of CyPD to PC and recruitment of HKII to mitochondria via HKII–VDAC interaction, GSK-3β–VDAC interaction, and phospho-GSK-3β–ANT interaction appear to modify Ca\(^{2+}\) sensitivity of the mPTP. Phospho-STAT3 and SIRT3 in the matrix are involved in signal-mediated and age-related changes in the mPTP sensitivity, respectively. Further clarification of both regulatory mechanisms of the mPTP and their impairments by pathological signalling is necessary to develop therapy directly targeting the mPTP for the management of ischaemic heart disease and heart failure.

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