Chapter 7

Protein Kinases and Regulation of Mitochondrial Function in Ischemia/Reperfusion Injury

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Additional information is available at the end of the chapter

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Abstract

Ischemic heart disease and stroke are the leading causes of death worldwide. Nonetheless, our understanding of the molecular mechanisms regulating cardiac and cerebral ischemic injury is very modest and our ability to develop therapies arresting and/or reversing detrimental events that spread from the ischemic core to the surrounding tissue is limited. Ischemia occurs when oxygen is unavailable to tissues due to occlusion of an artery (myocardial infarction, stroke, and pulmonary embolism), hemorrhage, organ transplantation, or hypotension in septic shock. The mitochondrion is a key target of ischemia. Alterations in mitochondrial morphology, dynamics, and functions result in energy deficits and contribute to the pathogenesis of ischemic injury. Phosphorylations of mitochondrial proteins and protein kinases that mediate them are important regulators of mitochondrial functions and tissue ATP levels. Thus, mitochondrial protein kinases could serve as targets for therapeutic interventions to mitigate the effects of ischemic injury. This will review the mitochondrial proteins regulated by phosphorylation, protein kinases mediating these reactions, and their implications in mitochondrial functions in ischemia/reperfusion (I/R)-induced injury.

Keywords: protein kinases, phosphorylation, mitochondria, mitochondrial dysfunction, ischemia, reperfusion, injury, ATP, oxidative phosphorylation, electron transport chain

1. Introduction

Ischemia (insufficient oxygen and nutrient supply to an organ) can affect all major organs and is often encountered in many clinical and nonclinical settings including myocardial infarction, stroke, pulmonary embolism, major surgery, tissue trauma and hemorrhage, transplantation

and organ storage, or hypotension in septic shock. Functional deficits caused by ischemia in major organs result in significant morbidity, disability, and mortality. Mitochondria are double-membrane-bound, dynamic organelles present in most eukaryotic cell types. They require large amounts of oxygen to generate ~90–95% of the total energy in oxidative phosphorylation occurring in a majority of cells [1]. Therefore, mitochondria are the key subcellular targets of ischemia, which undergo pathological changes that trigger cellular and tissue damage when oxygen is unavailable. Mitochondria perform several major cellular functions including energy and intermediary metabolisms, several biosyntheses, regulation of calcium storage, and redox homeostasis [1]. They are also involved in signaling, cell cycle, growth, differentiation, and cell death by apoptosis [2].

2. Major mitochondrial targets of ischemia/reperfusion (I/R)

Proteins of the mitochondrial electron transport chain and oxidative phosphorylation are among the primary targets of ischemia and oxidative stress during reperfusion [3]. Decreases in activities of adenine nucleotide translocase (ANT) and ATP synthase are among the earliest events after the onset of cardiac ischemia [4–7]. Inhibition of NADH:ubiquinone dehydrogenase (complex I) and reduced cytochrome c content occur early during ischemia [5, 8], whereas the damage to ubiquinol:cytochrome c oxidoreductase (complex III) and cytochrome oxidase (complex IV) occurs in prolonged ischemia [5, 8]. Reduced activity of complex I is primarily caused by decreases in NADH dehydrogenase activity [5, 9] due to, in part, oxidative damage to the flavin mononucleotide (FMN) prosthetic groups, which results in electron leakage, superoxide production, and the generation of reactive oxygen species (ROS) in ischemic tissues [5, 10]. Ischemia decreases the activity of complex III by inactivating the iron-sulfur center, which contributes to electron leakage and superoxide production, and exacerbates oxidative stress originating from complex I [11]. The voltage-dependent anion channel (VDAC) and proteins of the mitochondrial permeability transition (MPT) pore are also targeted by I/R, which disrupts the transport of ions and solutes and the membrane potential for ATP synthesis [12, 13].

Mitochondria are dynamic organelles that regularly undergo fission (fragmentation) and fusion (formation of a network of mitochondria) [14]. Fission is mediated by the dynamin-related protein 1 (Drp1) and mitochondrial fission protein 1 (Fis1) [14]. Fusion is regulated by the mitofusins (Mfn1 and Mfn2) and the optic atrophy protein 1 (Opa1) [14]. Drp1 and Opa1 are rapidly activated and translocated to mitochondria after ischemia in major organs. This promotes fission, the permeabilization of the mitochondrial outer membrane, and the release of proteins that initiate the intrinsic cascade of apoptosis [15, 16]. Inhibition of Drp1 inhibits fission and reduces cardiomyocyte death, the size of myocardial infarct, and acute kidney injury after ischemia [17]. Mitochondria also regulate the intrinsic cascade of apoptosis, which is activated after the release of mitochondrial proteins. Therefore, preservation of mitochondrial integrity and function is crucial for organ protection against I/R injury.

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3. Phosphorylation of mitochondrial proteins as a regulatory mechanism of energy metabolism during ischemia/reperfusion

Numerous mitochondrial proteins (354 reported to date) are phosphoproteins that collectively contain 899 identified and 479 potential novel phosphorylation sites [18, 19]. Consequently, phosphorylation of mitochondrial proteins has emerged as an important mechanism involved in progressive damage of mitochondria in response to metabolic stresses including I/R and the status of these phosphorylations is key to understanding the regulation of mitochondrial functions in disease states [18, 19]. A number of protein kinases localize to mitochondria in response to I/R [19, 20]. Protein phosphorylations by these kinases produce differential outcomes in different tissues depending on the phosphorylation site and the kinase involved. The key proteins of oxidative phosphorylation, TCA cycle, transport, and the cascade of intrinsic apoptosis are regulated by phosphorylation [19–23].

3.1. Phosphorylation of proteins involved in oxidative phosphorylation

The largest group of phosphorylation sites was found among proteins involved in oxidative phosphorylation: respiratory complexes, ATP synthase, ANT, and VDAC [18, 19].

4. NADH-ubiquinone oxidoreductase (complex I)

Dysfunction of complex I is the most common disorder of oxidative phosphorylation in humans. It is often due to defects of the subunit assembly to form the mature complex I. Complex I is a major mitochondrial target of I/R. Its activity decreases as early as 10 minutes into cardiac ischemia [24]. Our studies demonstrated that the activity of complex I in renal cortical mitochondria is decreased after renal ischemia [25]. Changes in the activity of complex I during I/R are also regulated by phosphorylation [4, 26]. Phosphorylations occur on several subunits of complex I. The NDUFA10 subunit is phosphorylated on S59 and S95 [27]. The NDUFS4 and NDUFA10 subunits are phosphorylated by mitochondrial protein kinase A (PKA), which stimulates the activity of complex I [27–29]. Phosphorylations of subunit ESSS on S20 and subunit MWFE (NDUFA1) on S55 regulate complex I assembly. Blocking these phosphorylations inhibits assembly of subunits to form a mature complex I and reduces its activity [26, 30]. Tyrosine phosphorylation of the NDUFB10 subunit by Src kinases also increases the activity of complex I, possibly by increasing its affinity toward NADH or increasing assembly of subunits into the fully active complex I [31, 32]. The reduced assembly of subunits leads to decreased levels of complex I [32]. This adaptation shifts fuel utilization from fuels that generate primarily NADH (carbohydrates) to fuels generating more $FADH_2$ (fatty acids) oxidized by complex II. This shift may occur during nutritional restriction or after ischemic injury [32]. Further, complex I is primarily assembled into mitochondrial super-complexes, which increases O_2 consumption and reduces ROS production [33]. Cardiac I/R induces disintegration of mitochondrial

super-complexes, which reduces activity of the electron transport chain [34]. Thus, phosphorylation controls the formation, stability, and function of complex I and its assembly into super-complexes.

5. Succinate-ubiquinone oxidoreductase (complex II)

Complex II is an essential regulator of metabolic reprogramming and respiratory adaptation. Mitochondrial Src-type tyrosine kinase Fgr phosphorylates complex II on Y535, Y596, and Y604 when activated by ROS generated by I/R [32]. Phosphorylation of Y604 on the flavoprotein subunit of succinate dehydrogenase (FpSDH) increases activity of complex II and serves as a metabolic adaptation to increased ROS production [32, 34]. Fgr-mediated phosphorylation also reduces the protein levels of complex I, which alters the mitochondrial preference for fuel oxidation from NADH to $FADH_2$, which increases the metabolic capacity of mitochondria to utilize alternative fuels when complex I is impaired [32]. Blocking phosphorylation of FpSDH on Y604 abolishes the capacity of mitochondria to adapt their metabolism after hypoxia/reoxygenation [32]. Mitochondrial phosphatases dephosphorylate Y604 and reverse this metabolic adaptation [32, 34]. In contrast, phosphorylation of FpSDH in cancer cells undergoing hypoxia decreases and dephosphorylation of FpSDH increases SDH activity [35]. Our data show that the activity of complex II in injured renal proximal tubular cells (RPTC) and in the ischemic kidney cortex is unchanged, whereas the activity of complex I is decreased [25, 36]. Supplementing the RPTC with succinate (complex II substrate) ameliorates mitochondrial dysfunction, ATP deficits, oxidative stress, and cell death after injury associated with the generation of ROS and oxidative stress [36].

6. Ubiquinol-cytochrome c oxidoreductase (complex III)

Phosphorylation has been implicated in the regulation of the Rieske iron-sulfur protein of complex III, which is a major target of ischemia and the decreases in its activity lead to increased superoxide production [37]. Several phosphorylation sites have been identified on the subunits of complex III. The tyrosines on the core subunit 1 of complex III are phosphorylated by the Src kinase family, but the functional consequence of this phosphorylation is not yet known [38]. The role of phosphorylation of Rieske iron-sulfur protein is not clear and it was suggested that it regulates the MPTP opening [37].

7. Cytochrome oxidase (complex IV)

To date, 14 phosphorylation sites have been mapped on complex IV [39]. Tyrosine phosphorylation of the specific subunits of complex IV can lead to both inhibition and activation of complex IV activity [39, 40]. Bender and Kadenbach have shown phosphorylation of complex IV subunits I, II/III, and Vb *in vitro* [41]. cAMP-dependent phosphorylation of Y304 on the catalytic subunit I inhibits, whereas tyrosine phosphorylation of subunit II by

c-Src kinase activates complex IV [41, 42]. The latter event is required for the normal function of cells, which are dependent on the efficient production of ATP to maintain their functions [42]. Interestingly, phosphorylation of the same subunit by the receptor tyrosine kinase ERBb2 decreases the activity of complex IV and mitochondrial respiration [43, 44]. Complex IV activity is inhibited in a time-dependent manner after myocardial ischemia, which stimulates multiple phosphorylations of complex IV: (1) subunit I on S115 and S116, (2) subunit IVi1 on T52, and (3) subunit Vb on S40 in the heart [38, 45]. These inhibitory phosphorylations are mediated by PKA and inhibition of PKA reduces I/R injury to the myocardial tissue [45]. Phosphorylation of subunit IV-1 on S58 by PKA increases the activity of complex IV by preventing allosteric inhibition of complex IV by ATP [46]. It was proposed that phosphorylation of S58 switches mitochondrial metabolism from energy utilization to energy storage in pathological conditions including I/R-induced injury [46].

8. Cytochrome c

Phosphorylations of serine, threonine, and 2 tyrosine residues have been mapped on cytochrome c [39, 47]. Phosphorylation of T28 results in a partial inhibition of the electron transport chain and respiration [48]. It was suggested that the other phosphorylations regulate the mobility of cytochrome c between complexes, its binding to cardiolipin, and the interaction with Apaf-1 during apoptosis [47].

9. ATP synthase (F_oF_1-ATPase)

Multiple and differential phosphorylations of ATP synthase have been reported in different organisms and tissues. Tyrosine phosphorylations of the ε-subunit (in the F_o domain) and the α- and δ-subunits of the F_1 domain are mediated by Src kinase [49, 50]. The α and e subunits of F_oF_1-ATPase in mammalian brain are phosphorylated on S76 and Y32 [51]. The catalytic β-subunit is extensively phosphorylated on S106, T107, T262/S263, T312, and T368 in mammalian cardiomyocytes, whereas in yeast, the β-subunit is phosphorylated on T58, S213, T262, and T318 [51]. These phosphorylations affect assembly of the F_1 domain and reduce ATP synthase activity [51]. They occur in cardiac preconditioning, which offers protection against ischemia [51]. Phosphorylation of T213 on the β-subunit of skeletal muscle F_oF_1-ATPase downregulates its levels *in vivo* [6, 51]. We have shown serine phosphorylations on the α and/or β- and γ-subunits of ATP synthase in RPTC [52]. These phosphorylations are PKC-α-dependent and preserve the levels of the γ-subunit and F_oF_1-ATPase activity in injured renal cells [52]. Phosphoproteomics demonstrated phosphorylation of S146 on the γ-subunit [52]. Phosphorylation of the γ-subunit is associated with the formation of ATP synthase dimers [52].

10. Adenine nucleotide translocase (ANT)

ANT, an antiporter embedded in the inner mitochondrial membrane that facilitates the exchange of ADP and ATP, is one of the most abundant mitochondrial proteins and a primary

target of ischemia [5, 53]. The four human isoforms, ANT1, 2, 3, and 4, are phosphorylated on tyrosines [49]. Phosphorylation of ANT on Y194 and Y190 alters the activity of ADP/ATP translocase and the transport of both nucleotides in the brain [49]. Phosphorylation of ANT1 at Y194 in cardiac tissue is diminished by I/R but maintained by pre- and post-conditioning, which suggests that this phosphorylation plays a protective role against ischemia in the heart and could improve tolerance against injury [54]. Although this phosphorylation improves respiration and mitochondrial function, it is not known whether phosphorylation of Y194 is sufficient for protection of cardiomyocytes from I/R-induced injury [54]. A binding of phosphorylated (inactive) glycogen synthase kinase-3β (GSK-3β) to ANT was also shown, but it is unclear whether GSK-3β directly phosphorylates ANT [55].

11. Voltage-dependent anion channel (VDAC)

VDAC, a channel protein localized to the outer mitochondrial membrane, conducts a variety of small metabolites (NAD^+/NADH, ADP, and ATP) and ions across the outer membrane and is a key regulator of energy metabolism [56]. VDAC forms a complex with ANT, which facilitates influx of ADP into mitochondria and the efflux of ATP to the cytoplasm [56–58]. VDAC1 closure leads to hyperpolarization of mitochondria, disruption of ADP/ATP exchange, decrease in ATP synthesis, and metabolic dysfunction, and rupture of mitochondria [56–58]. The four known VDAC isoforms present in the outer mitochondrial membrane are phosphorylated on multiple serines and threonines [21, 22]. A total of 19 distinct phosphorylations were identified in VDAC isoforms [21, 65]. These phosphorylations are mediated by different kinases including PKA, PKC, tyrosine kinase, hexokinase, GSK-3β, Akt, JNK3, and p38 [59]. The phosphorylation of neuronal and hepatic VDAC1 by PKA and ischemia-activated GSK-3β and JNK induces its closure, disrupts formation of the complex with ANT, and decreases ATP synthesis [59]. In contrast, phosphorylation of cardiac VDAC1 by protein kinase C-ε (PKC-ε) promotes formation of the VDAC-ANT complex, prevents the opening of MPTP, and protects mitochondrial integrity after I/R injury [60]. Phosphorylation of VDAC1 by never-in-mitosis A related kinase 1 (Nek1) on S193 closes the channel in RPTC, blocks release of cytochrome c, and prevents cell death by apoptosis [61]

11.1. Phosphorylation of enzymes of the tricarboxylic acid cycle (TCA)

Phosphoproteomic analyses revealed that most enzymes of the TCA cycle are phosphorylated/dephosphorylated [62]. These include aconitase, isocitrate and oxo-ketoglutarate dehydrogenases, succinyl-coenzyme A synthetase, succinate dehydrogenase, fumarate hydratase, and mitochondrial malate dehydrogenase [62, 63]. Although the functional role of these phosphorylations and the protein kinases that mediate them are not yet known, data suggest that phosphorylation regulates the activity of these enzymes.

11.2. Pyruvate dehydrogenase complex (PDC)

PDC is composed of multiple copies of three distinct enzymes: (1) pyruvate dehydrogenase (E1), dihydrolipoyl transacetylase (E2), and dihydrolipoyl dehydrogenase (E3), which form

a large complex. PDC is the rate-limiting enzyme in the oxidative metabolism of all carbohydrates and its activity is tightly regulated by multiple mechanisms including phosphorylation. Phosphorylation of the PDC by the pyruvate dehydrogenase kinase (PDK) inactivates the complex [64]. The α-subunit of the E1 is phosphorylated at multiple sites, but the most known are phosphorylations of three distinct serines [64]. Although phosphorylation at any of these serines is sufficient to inhibit the activity of PDC, the S293 residue has the highest affinity for phosphate, and phosphorylation of this serine has the greatest impact on the inhibition of activity of PDC [64, 65]. I/R-induced injury in cardiac tissue is associated with a 4–5-fold decrease in the phosphorylation of S293, which results in the activation of PDC [64]. PDC activation protects against ischemic injury and improves cardiac efficiency and contractile capacity in the postischemic heart [65].

11.3. Proteins involved in mitochondrial dynamics

Mitochondrial morphology is dynamically changed by the balance between fusion and fission (fragmentation). Phosphorylations of GTPases, Drp1, Mfn1/2, and Opa1 regulate mitochondrial fission and fusion [66]. Phosphorylation of fission-inducing Drp1 on S616 and S637 can be mediated by cyclin-dependent kinase (cdk)-1, cdk-5, PKA, PKC-δ, and extracellular signal regulated kinase-2 (ERK-2) [66–71]. Phosphorylation of Drp1 at S616 by cdk-1, PKC-δ, and ERK-2 promotes Drp1 translocation to mitochondria and fission whereas phosphorylation of S637 by PKA inhibits Drp1 and mitochondrial fission [67–71]. We have demonstrated that activation of PKC-ε induces the translocation of Drp1 to mitochondria, mitochondrial fission and apoptosis in non-injured RPTC [72]. Drp1-dependent mitochondrial fission is associated with mitochondrial outer membrane permeabilization and apoptosis, whereas elongation of the mitochondria through fusion promotes ATP synthesis and prevents mitochondrial autophagy. When phosphorylated by ERK, Mfn1 has decreased ability to oligomerize and tether mitochondria, which inhibits their fusion, recruitment of Bak to the mitochondria, and apoptosis [73]. Mfn2 is phosphorylated on T111 and S442 by PTEN-induced putative kinase protein 1 (PINK1), which recruits the protein Parkin to depolarized mitochondria and eliminates them by mitophagy [74]. Disruption in the balance between fission and fusion occurs in pathological conditions including I/R, which increases phosphorylation of Drp1, its translocation to mitochondria, and mitochondrial fission in the heart and brain [75]. Inhibition of Drp1 protects the heart and brain against ischemia and has been proposed as a therapeutic target following cardiac arrest [76].

11.4. Proteins of the intrinsic apoptotic cascade

Mitochondrial proteins involved in the intrinsic apoptosis include Bcl-2 family members, AIF, Smac/DIABLO, cytochrome c, and Omi/Htra2. With an exception of one, all are regulated by phosphorylation [20]. Phosphorylation of Bcl-2 at S70 is required to prevent permeabilization of the mitochondrial outer membrane and for Bcl-2's anti-apoptotic activity [77]. Several protein kinases serve as Bcl-2 kinases to inhibit (JNK, p38, and GSK-3) or activate (Akt, PKA, and PKC-α) Bcl-2 [77]. Cardiac and neuronal tissues are protected against I/R when Bcl-2 is active [78–80]. Ischemia-induced acute kidney injury upregulates Bcl-2 and Bcl-XL in the distal and Bax in the proximal tubules [80]. Pro-apoptotic Bax, Bak, Bad, and Bid are phosphorylated on serines and tyrosines, which controls their insertion into the outer mitochondrial membrane

and formation of pores that mediate the release of pro-apoptotic AIF, Smac, and cytochrome c from the mitochondria [81]. Smac is phosphorylated by JNK3, which decreases its pro-apoptotic actions [81]. Phosphorylation of Y97 and Y48 on cytochrome c regulates its capacity to form the apoptosome and activate caspase-9 [81]. Thus, phosphorylation of specific amino acids on a mitochondrial protein determines the fate of mitochondria by protecting their integrity and functions or inducing MPT and apoptosis.

12. Role of protein kinases in regulating mitochondrial functions in ischemia/reperfusion

Ischemia rapidly changes activities of different protein kinases including the calcium/calmodulin-dependent protein kinase II (CaMK-II), PKA, protein kinase B (PKB/Akt), PKC, Raf-1, ERK1/2, c-jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38 MAPK), GSK3-β, PINK1, and tyrosine kinases, which suggests their involvement in I/R injury.

12.1. Protein kinases regulating large dehydrogenase complexes

A specific family of protein kinases is exclusively present in the mitochondrial matrix of eukaryotic cells. These kinases have different sequences from the cytosolic protein kinases and they phosphorylate and inactivate large enzymatic complexes in the mitochondrial matrix, the branched-chain α-ketoacid dehydrogenase complex and pyruvate dehydrogenase complex, PDC. Perturbations in PDC activity result in energy deficits, neuronal dysfunction, and brain injury, such as those observed in stroke. Phosphorylation of the E1 α-subunit of PDC by PDK isozymes inhibits, whereas dephosphorylation increases PDC activity [82]. Cardiac and cerebral ischemia has no effect on the activity of PDC; however, reperfusion results in a fast decline in PDC activity in the brain [83]. Increased expression of PDK2 following traumatic brain injury may maintain E1 in the hyperphosphorylated (inactive) state, which impairs glucose oxidation after stroke and traumatic brain injuries [83]. Further, reperfusion-induced oxidative stress activates PKC-δ, which translocates to mitochondria and activates PDK2 leading to inhibition of E1 activity [84]. The inhibition of PDK during reperfusion restores PDC activity and decreases brain injury, demonstrating that phosphorylation by PDK2 mediates inhibition of PDC activity [85]. Infusion of the specific peptide inhibitor of PKC-δ, Tat-δV1-1, prevents the translocation of PKC-δ to mitochondria and maintains PDC activity [84]. Isozymes of PDK are considered attractive targets for therapies to improve PDC activity to diminish the detrimental effects of I/R in tissues that depend on metabolism of glucose, such as neuronal and cardiac tissues.

12.2. Serine/threonine kinases in regulation of mitochondrial functions in ischemia/reperfusion

12.2.1. Protein kinase A

PKA is activated by a signaling pathway originating from G-protein receptors, which produces cAMP. The association of PKA with mammalian mitochondria was documented four

decades ago [86]. Mitochondrial PKA is present in the outer and inner mitochondrial membranes as well as in the mitochondrial matrix [28, 87]. Several mitochondrial substrates of PKA with distinct tissue-specific responses have been identified. During myocardial I/R, PKA is activated by increased levels of ROS and translocates to mitochondria [88]. The increase in activity of mitochondrial PKA is independent of mitochondrial levels of cAMP and is due to increased sequestration of the catalytic α-subunit in the mitochondrial matrix [88]. The presence of the catalytic α-subunit of PKA results in phosphorylation of subunits I (on S115 and S116), IVi1 (on T52), and Vb (on S40) of complex IV, followed by their degradation and the loss of activity [38, 40, 88]. Because the identified phosphorylation sites on complex IV are not within PKA consensus sites, it was proposed that PKA activation plays an indirect role in the loss of complex IV activity during myocardial ischemia [39]. These changes are sufficient to disrupt the function of mitochondrial respirasome and increase production of ROS [88]. PKA-mediated phosphorylation regulates ischemia-induced dysfunction of complex IV [38, 45, 88]. Ca^{2+} influx activated by PKA in cardiomyocytes induces mitochondrial permeability transition, which stimulates caspase-9 and apoptosis [89]. Inhibition of PKA prevents the loss of activity of complex IV during cardiac I/R, promotes postischemic cardiac contractile recovery, and decreases the infarct size in the ischemic heart [38, 45, 90]. Thus, PKA inhibitors could serve as candidates for cardioprotective agents [87, 89]. PKA also phosphorylates NDUFS4 and NDUFA10 subunits of complex I and this phosphorylation stimulates the activity of complex I and complex I-driven respiration [28, 29, 91]. Finally, Hsp20, which localizes to mitochondria and is expressed at high levels in cardiac, skeletal, and vascular smooth muscle, is regulated by the β-adrenergic/PKA signaling pathway [92]. PKA-mediated phosphorylation of Hsp20 on S16 increases in ischaemic myocardium and is cardioprotective [92].

In contrast, the activation of PKA during liver IR is cAMP-dependent and plays a protective role against liver injury [93]. The cAMP that activates mitochondrial PKA does not originate from membrane-bound adenylyl cyclase activated by the Gα protein, but is produced inside mitochondria by the carbon dioxide/bicarbonate-regulated soluble adenylyl cyclase in response to metabolically generated carbon dioxide [93]. Mitochondrial PKA regulates mitochondrial biogenesis, normalizes ROS production, and activates complex IV [46]. Inhibition of PKA exacerbates hepatocellular damage, whereas increasing cAMP levels to activate PKA protects the ischemic liver from injury [93]. PKA activation diminishes neutrophil and macrophage infiltration into ischemic liver tissue, reduces production of tumor necrosis factor α, interleukin (IL)-6, and IL-12 by macrophages, increases IL-10 expression, and prevents hepatocyte death [93]. Thus, PKA activation reduces the inflammatory response associated with reperfusion after liver ischemia. Because ischemia is an inherent component of liver transplantation, activation of PKA was proposed as a rationale for novel therapies to combat I/R injury and protect transplants [94]. However, increased cAMP levels in the liver *in vivo* inhibit complex IV due to phosphorylation of Y304 [41]. Because PKA is not a tyrosine kinase, it was proposed that PKA activates a downstream tyrosine kinase, which phosphorylates and inactivates complex IV [41]. Therefore, attempts aimed at activating PKA to protect the liver against ischemia should be treated with great caution.

PKA dysregulation has been implicated in several neurodegenerative disorders. PKA-dependent pathways in different regions of the brain play a role in pathogenesis and cognitive decline in Alzheimer and Parkinson's diseases [94]. The acute phase of cerebral ischemia

is accompanied by decreases in affinity of PKA for cAMP and PKA activity [94]. In contrast, the peri-ischemic and less-injured areas exhibit increased PKA-mediated phosphorylation compared to the ischemic core, which suggests that active PKA is associated with the survival of neuronal tissue [94]. Derangement of cAMP-dependent signal transduction is associated with ischemic neuronal damage and activation of the PKA is important for neuronal survival in acute cerebral ischemia [94]. Phosphorylation of Bad at S155 by mitochondria-anchored PKA leads to cytosolic sequestration of Bad and blocks its mitochondrial translocation, the release of cytochrome c, and neuronal apoptosis [77]. Also, PKA-mediated phosphorylation of Drp1 at S637 blocks its translocation to mitochondria and their fission and promotes mitochondrial fusion. In hippocampal neurons, expression of a constitutively active catalytic subunit of PKA targeted to the mitochondrion promotes the fusion of mitochondria into networks [95]. Finally, PKA suppresses autophagy and mitochondrial degradation in neurons by phosphorylating and inhibiting the microtubule-associated protein 1A/1B-light chain 3 (LC3) and increases neurite outgrowth [94, 96].

12.2.2. Protein kinase B (PKB/Akt)

Akt can localize to the mitochondrial matrix and the inner and outer membranes of cardiac, neuronal, and kidney cells [97, 98]. Protein levels of mitochondrial Akt are regulated by a variety of extracellular signals and stresses, which induce rapid translocation of active (phosphorylated) Akt to the mitochondria [97, 98]. Mitochondrial localization of Akt is associated with cardioprotection against ischemic injury and renoprotective actions against chemical toxicity [97–100]. The cardioprotective effect of mitochondrial Akt is attributed to its action on mitoK(ATP) channels and to decreasing apoptosis [99]. Akt phosphorylates pro-apoptotic Bad on S136 and Bax on S184, and prevents MPT in neuronal cells [101]. Phosphorylated Bad associates with 14-3-3 proteins in the cytosol and cannot form complexes with mitochondrial Bcl-2/Bcl-XL to induce permeabilization of the mitochondrial outer membrane and MPT [101]. Bax phosphorylation by Akt promotes dimerization of Bax with Bcl-XL or Mcl-1 proteins, which sequesters Bax away from mitochondria and prevents their permeabilization and apoptosis [101]. Akt prevents the MPT in cardiomyocytes by phosphorylating hexokinase II, stabilizing it in the outer mitochondrial membrane, and promoting its binding to VDAC [102]. Inhibition of Akt or targeted disruption of the association of hexokinase II with mitochondria abolishes cardioprotection [102].

We have identified mitochondria as a subcellular target of protective actions of Akt against necrosis in injured RPTC [98]. Mitochondrial levels of active Akt decrease in injured RPTC and this is associated with mitochondrial dysfunction [98, 100]. Selective activation of Akt increases the levels of Akt in mitochondria, improves state 3 respiration, activities of complexes I and III and F_0F_1-ATPase (ATP synthase), and the mitochondrial membrane potential ($\Delta\Psi_m$), increases ATP production, and reduces ATP deficits [100]. Selective inhibition of Akt exacerbates mitochondrial dysfunction, energy deficits, and necrosis in injured RPTC [100]. These results are consistent with a report that Akt phosphorylates the β-subunit of ATP synthase and improves its activity and increases ATP production to prevent energy deficits in injured cells [97]. Also, active Akt increases activity of PDC and oxidative metabolism of carbohydrates by inhibiting phosphorylation of PDC by GSK-3β [103]. Thus, signals from cell

membrane receptors or those generated by metabolic stress have a rapid effect on Akt activation status in mitochondria. Akt could serve as a therapeutic target promoting mitochondrial functions in ischemic organs. However, the application is complicated by the fact that Akt promotes tumor formation by blocking apoptosis.

12.2.3. Protein kinase C

PKC plays a key role in mediating I/R injury in the brain, heart, and kidneys; however, elucidation of this role is complicated due to the presence of 11 distinct PKC isozymes, which have unique tissue and cellular localizations and often play quite opposite roles in ischemic injury even within the same organ. Alterations in PKC activity or subcellular localization during ischemia occur in the brain, heart, liver, and kidney [25, 104–106]. Major isozymes of PKC (α, β, γ, δ, ε) translocate to mitochondria in response to ischemia and/or reperfusion and oxidative stress, and have been implicated in regulating mitochondrial functions in ischemic and postischemic tissues [104]. The classical PKC isozymes (α, β, γ) are dependent on Ca^{2+} and 1,2-diacylglycerol, and ischemia increases the levels of both activators through the stimulation of phospholipase C. PKC-α activation has been implicated in ischemia-induced heart failure and cerebral barrier breakdown after ischemic stroke [107, 108]. PKC-α inhibition or deletion protects the heart from decompensation and cardiomyopathy and attenuates cerebral barrier breakdown after ischemia [107, 108]. In contrast, PKC-α translocation to mitochondria is protective against mitochondrial dysfunction, ATP deficits, and cell death caused by ischemia, hypoxia, and oxidative stress in RPTC [109]. We have shown that ATP synthase (F_0F_1-ATPase) is a target of PKC-α, which associates with the α-, β-, and γ-subunits of the F_1 domain of ATP synthase [52]. Injury or inactive PKC-α disrupt, whereas active PKC-α promotes this association and increases the levels of α-, β-, and γ-subunits in injured RPTC [52]. Active PKC-α promotes phosphorylation of the γ-subunit on S146 and F_0F_1-ATPase activity after injury [52]. PKC-α also reduces apoptosis by phosphorylating mitochondrial Bcl-2 on S70 [77, 110]. Thus, activation of PKC-α exerts differential effects in different types of cells and tissues.

PKC-γ is expressed exclusively in neurons of the brain and spinal cord and is activated rapidly during ischemia and inhibited during reperfusion [111–114]. PKC-γ plays a detrimental role in the early stages of ischemia mediating events leading to cell death. PKC-γ knockouts show smaller infarct areas after cerebral ischemia [112]. Also, PKC-γ may directly (or through the activation of a Src tyrosine kinase) phosphorylate NMDA receptors, stimulate their function, and increase the concentration of intracellular Ca^{2+}, which results in mitochondrial dysfunction, ATP deficits, increased ROS formation, and neuronal death [112]. However, after reperfusion, PKC-γ may mediate protection against cell death [112]. Thus, the same PKC isozyme may play opposing roles at different stages of ischemic injury.

PKC-δ is rapidly activated by signaling initiated by reperfusion. PKC-δ plays a detrimental role in ischemic stroke injury and mediates oxidative stress, cell death, and inflammation associated with reperfusion [111, 112]. Selective inhibition or deletion of PKC-δ reduces infarct size and ischemic brain injury caused by middle cerebral artery occlusion, specifically, reperfusion-induced death of parenchymal cells [115, 116]. PKC-δ inhibition activates PKB/Akt, inhibits translocation of Bad to mitochondria, and decreases apoptosis [112]. PKC-δ

mediates neutrophil infiltration, which is responsible for the detrimental effects of PKC-δ during reperfusion [112]. Similarly, reperfusion after cardiac ischemia activates PKC-δ, which translocates to mitochondria and reduces state 3 respiration, TCA cycle, and ATP production, increases generation of mitochondrial ROS, and induces release of cytochrome c and cell death [84, 113, 115, 116]. Selective inhibition of PKC-δ translocation to mitochondria blocks these changes and protects the heart from ischemic injury [117]. PKC-δ is also activated by oxidative stress in the kidney, exacerbates RPTC and kidney injury by activating Bax, inducing cytochrome c release and apoptosis, and blocking autophagy. PKC-δ inhibition protects kidneys from injury by upregulating autophagy [118].

PKC-ε, another novel isozyme, is activated and translocates to mitochondria during I/R. These events have been implicated in the cardio- and neuro-protection against ischemic injury and in the reduction of myocardial infarct [119, 120]. PKC-ε activation is a pivotal signaling event in the cardioprotective mechanisms of ischemic preconditioning, and it is thought that this protection is mediated through mitochondrial and transport mechanisms [120]. Conversely, inhibition of PKC-ε eliminates the cardioprotection [120]. Several substrates of PKC-ε are present in cardiac mitochondria. Active PKC-ε induces opening of the mitoK(ATP) channels, maintains ATP production, and reduces ROS production [120]. Specifically, PKC-ε regulates interactions between conexin43 and the mitoK(ATP) subunit, which leads to mitoK(ATP) opening when ATP levels decrease, resulting in cardioprotection [121]. Active PKC-ε also increases phosphorylation of subunit IV and the activity of complex IV [122]. Thus, the PKC-ε-mediated resistance to cardiac ischemia may also be due to increased activity of the electron transport chain and a greater $\Delta\Psi_m$ for ATP synthesis. Also, PKC-ε phosphorylates VDAC1 on T51, which modifies its gating and interaction with proteins of the MPT pore to inhibit its opening and maintain $\Delta\Psi_m$ and ATP synthesis [60]. These data support protective and prosurvival actions of mitochondrial PKC-ε in cardiac I/R. In contrast, translocation of the active PKC-ε to mitochondria in RPTC results in mitochondrial dysfunction, decreases in ATP levels, mitochondrial fission, and RPTC death [25, 72]. Active PKC-ε produces the classical hallmarks of mitochondrial dysfunction in RPTC: decreases in state 3 respiration and activity of complex I, increases in $\Delta\Psi_m$, ROS production, and mitochondrial fission [25, 72]. Inhibition of PKC-ε protects against mitochondrial dysfunction induced by hypoxia and oxidative stress [72]. Deletion of PKC-ε *in vivo* ameliorates I/R-induced decreases in respiration and activities of complexes I, III, and IV, and reduces oxidant production and morphological damage in ischemic kidneys [25]. Deletion of PKC-ε reduces the inflammatory response and apoptosis, and promotes renal function and survival after I/R-induced acute kidney injury in mice [25, 123]. Thus, in contrast to the cardio- and neuroprotective effects of PKC-ε, activation of this PKC isozyme in the renal cortex is detrimental to mitochondria, cell viability, and kidney functions [25, 123].

12.2.4. Mitogen-activated protein kinases (MAPK)

ERK1/2 localize to the mitochondria of cardiac, brain, and renal epithelial cells. Brain and cardiac ischemia activates ERK1/2, which form signaling modules with PKC-ε and translocate to mitochondria [124–126]. ERK1/2–PKC-ε modules play a role in the phosphorylation and inactivation of Bad, which blocks the intrinsic pathway of apoptosis [126]. Furthermore, active

ERK1/2 associates with the outer mitochondrial membrane and protects against MPTP opening and mitochondrial depolarization [127]. These events are thought to contribute to the cardioprotective effects of ERK1/2 activation against I/R injury. In contrast, ERK1/2 activation is detrimental to mitochondria and cell viability in RPTC [128]. We have shown that ERK1/2 activation and translocation to mitochondria in response to oxidant injury mediates mitochondrial dysfunction and cell death in RPTC [128]. ERK1/2 activation mediates decreases in state 3 respiration, activities of aconitase of the TCA cycle and complex I of the electron transport chain, and ATP production in injured RPTC [128]. ERK1/2 inhibition restores respiration, complex I activity, $\Delta\Psi_m$ and ATP production, and decreases RPTC death [128]. Recently, it was shown that ERK1/2 downregulates mitochondrial function through the EGFR/ERK1/2/FOXO3a/1/PGC-1α pathway by phosphorylating the upstream regulators of PGC-1α and decreasing mitochondrial biogenesis [129]. Similarly, ERK1/2 activation mediates inflammatory changes, infiltration by neutrophils, apoptosis, and severe injury after ischemia in the lung [130].

JNK and p38 MAPK are activated and localize in cardiac mitochondria after ischemia or oxidant exposure and mediate mitochondria-initiated apoptosis [131]. p38 MAPK inhibition attenuates the loss of $\Delta\Psi_m$, mitochondrial swelling, and ultrastructural changes, reduces cardiomyocyte apoptosis and infarct size, and improves left ventricular function after ischemia [131, 132]. Inhibition of p38 MAPK also decreases phosphorylation of p53 and Bax expression and reduces cytochrome c release from mitochondria and the levels of active caspase 3 [132]. These data suggests that active p38 MAPK mediates apoptosis.

12.2.5. Glycogen synthase kinase

GSK3β activated by cardiac I/R docks to mitochondria, phosphorylates VDAC1, and leads to phosphorylation of ANT and cyclophilin D, and MPTP opening [55, 133]. This suggests that GSK3β plays a role in mitochondria-mediated apoptosis in cardiac tissue. GSK3β also regulates mitochondrial oxidative metabolism by phosphorylating and inhibiting PDC [65]. Activation enhances whereas inhibition of GSK3β activates mTOR pathway, inhibits mTOR-dependent autophagy, and reduces myocardial I/R injury [134]. Inhibition of GSK-3β also attenuates brain infarct volume after cerebral I/R-induced injury [135].

12.2.6. PTEN-induced kinase 1

The mitochondrial serine-threonine protein kinase, PTEN (phosphatase and tensin homolog on chromosome 10)-induced kinase 1 (PINK1) localizes to both mitochondrial membranes and regulates mitochondrial function and dynamics [136]. PINK1 phosphorylates mitofusin MFN2, a mitochondrial receptor for Parkin (a protein related to Parkinson's disease) [136]. PINK1 regulates mitochondrial dynamics in ischemic stroke and prevents damage to neurons by reducing mitochondrial translocation of Drp1 and fission, and preventing the collapse of $\Delta\Psi_m$ and ATP synthesis [137, 138]. Deletion of PINK1 causes defects in the turnover of proteins of the electron transport chain, impairs mitochondrial respiration and activity of complex I in cardiomyocytes, induces MPTP opening, decreases production of ATP, exacerbates oxidative stress, and increases the size of ischemic myocardial infarct, which suggests increased susceptibility to I/R injury in PINK1-deficient hearts [139]. Overexpressing PINK1

reduces these changes [139]. Thus, this study suggests mitochondrial PINK1 as a target for cardioprotection against ischemia in the heart.

12.3. Tyrosine kinases and regulation of mitochondrial functions in ischemia/reperfusion

Tyrosine phosphorylation is a crucial mechanism for regulating mitochondrial functions [19, 42]. Tyrosine kinases of the Src family (Lyn, Lck, c-Src, Fyn, and Fgr) localize to mitochondria by binding to specific anchoring proteins present in mitochondria [19, 42]. The primary known role of tyrosine phosphorylation is the regulation of the respiratory chain by c-Src [19]. Phosphorylations of NDUFB10 subunit of complex I at Y193, succinate dehydrogenase of complex II at Y215, and subunit II of complex IV on unknown tyrosine by Src increase activities of complexes I, II, and IV [19, 31, 42]. Targeting of c-Src to mitochondria enhances $\Delta\Psi_m$ and oxidative phosphorylation in a c-Src-dependent manner [19]. Src and Lck phosphorylate ANT1 at Y194 and reduce ischemic injury in preconditioned cardiac cells [54]. Mitochondrial Fgr kinase phosphorylates complex II, which increases complex II activity and regulates NADH/$FADH_2$ balance in mitochondria [32]. Epidermal growth factor receptor (EGFR) translocates to mitochondria when phosphorylated on Y845 by Src [43]. EFR interacts with and phosphorylates subunit II of complex IV thereby decreasing its activity and cellular ATP levels [19, 140].

In conclusions, translocation of protein kinases to mitochondria regulates mitochondrial functions in various disease states including ischemia and reperfusion in major organs such as the brain, heart, and kidneys. These findings suggest that these protein kinases can serve as potential effective therapeutic targets to maintain mitochondrial integrity and functions and prevent or reduce organ damage in these disease states.

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