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Mitochondrial Protein Phosphorylation as a Regulatory Modality: Implications for Mitochondrial Dysfunction in Heart Failure

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Abstract

Phosphorylation of mitochondrial proteins has been recognized for decades, and the regulation of pyruvate- and branched-chain α -ketoacid dehydrogenases by an atypical kinase/phosphatase cascade is well established. More recently, the development of new mass spectrometry-based technologies has led to the discovery of many novel phosphorylation sites on a variety of mitochondrial targets. The evidence suggests that the major classes of kinase and several phosphatases may be present at the mitochondrial outer membrane, intermembrane space, inner membrane, and matrix, but many questions remain to be answered as to the location, timing, and reversibility of these phosphorylation events and whether they are functionally relevant. The authors review phosphorylation as a mitochondrial regulatory strategy and highlight its possible role in the pathophysiology of cardiac hypertrophy and failure.

The balance of energy supply and demand plays a central role in the adaptation of the heart to challenges such as pressure overload, oxidative stress, ischemia, or diabetes. The problem ultimately boils down to whether the heart is able to maintain an adequate supply of adenosine-5'-triphosphate (ATP) to maintain ion gradients, support contraction, and rebuild cellular components. While "energy starvation" has been hypothesized to be a contributor to the transition from functionally compensated hypertrophy to heart failure (HF),^{1–3} the details of the metabolic remodeling process, and which aspects are compensatory or maladaptive, is of critical interest with respect to developing new therapies. Metabolic remodeling encompasses a number of potential mechanisms including (1) changes in energy substrate availability, (2) changes in the levels or transport of metabolic intermediates, (3) changes in gene expression of metabolic enzymes, (4) changes in the stability and localization of expressed proteins (eg, impaired trafficking, folding, or degradation), and finally (5) post-translational changes in regulatory modulation of enzymes or transporters that influence metabolic flux through a particular pathway. Regulation of mitochondrial function by post-translational modification is the principal topic covered in this review.

As of July 2011, the number of different annotated post-translational modifications (PTMs) in the UniProt Knowledgebase (version 2011_08; <http://www.uniprot.org/docs/ptmlist>) is

431, of which 118 are listed as covalent crosslinks between amino acids. This list is not comprehensive, because it does not contain sites of covalent modification of small proteins such as ubiquitination or SUMOylation. The number of PTMs and sites identified continues to grow thanks to advances in PTM-enrichment methods and the proliferation of mass spectrometers that possess ever-increasing mass accuracy and sensitivity. Indeed, the rate of data generation is so fast that many published site identifications have not yet been incorporated into the major protein databases. Determination of the functional implications of PTMs detected in mitochondria, on the other hand, proceeds at a much slower pace. Nevertheless, some PTMs important for the regulation of metabolic fluxes have been known for many years, and interesting new modifications (both new proteins and new types of modification) and mechanisms are being published on a daily basis. Of particular interest are those PTMs that are linked to disease processes, longevity, cell survival, and proliferation.

The definition of a PTM can be either narrowly restricted to rapid and reversible modifications that may play a regulatory role (eg, phosphorylation, nitrosylation, acetylation), often mediated by specific enzymes, or broadly extended to include nonenzymatic processes such as disulfide modifications, unintended oxidative damage, and proteolysis, which can affect protein levels, localization, or function. Both definitions are important to consider in terms of the mechanisms of pathophysiology, because the interplay between regulatory PTMs and modifications contributing to organelle dysfunction may be instrumental in determining the extent of disease progression.^{4,5}

With respect to the PTMs that are potentially relevant to the progression of HF, a comprehensive picture has not yet emerged, but new technologies are leading to an accumulation of novel mitochondrial targets of phosphorylation, nitrosylation, acetylation, and O-GlcNAcylation, many of which have been linked to significant effects on individual protein activity or global mitochondrial function. Owing to the volume and scope of the literature available on these topics, we chose to narrow the focus of this review to mitochondrial phosphorylation as a potential regulatory strategy, reviewing mitochondrial proteins known to be subject to kinase/phosphatase action, while at the same time attempting to divine new directions for investigation into the mechanisms of HF.

Metabolic Remodeling Associated With HF

There are two prevalent, yet contradictory, views regarding shifts in substrate utilization during the development of hypertrophy and HF. The first stems from early observations showing that failing hearts had an increase in anaerobic and aerobic glucose utilization at the expense of fatty acid oxidation^{6,7} and this has been confirmed in a number of studies since then.^{8–11} More recently, the shift towards increased glucose utilization has been interpreted as a recapitulation of the fetal energetic phenotype due to genetic reprogramming, primarily through decreased activity of the metabolic transcriptional coactivator PGC1 α ,^{12–14} which acts by forming transcriptional regulatory complexes with the peroxisomal proliferation activator receptors (PPARs) in conjunction with the retinoid X receptor, the estrogen response (orphan) receptor (ERR α), nuclear respiratory factor 1 (NRF1), and GA repeat-binding protein alpha (GABP).^{15–17} The shift in substrate utilization from fatty acids towards glucose oxidation has been suggested to be a compensatory response that improves

the economy of force production with hypertrophy,¹⁸ since it has been shown that the cardiac energetic efficiency is higher when the substrate is shifted from fatty acids to glucose,^{19,20} but this change may also impose a limit on energy production during later stages of HF.

The opposing view is that fatty acid oxidation is specifically increased while glucose oxidation is impaired with cardiac hypertrophy and failure. Increased fatty acid oxidation has been reported in failing human hearts^{21,22} and therapeutic strategies aimed at inhibiting fatty acid oxidation to enhance glucose utilization have been investigated. For example, inhibition of carnitine palmitoyl transferase (CPT 1),²³ β -oxidation enzymes, or malonyl CoA decarboxylase^{20,24–27} has been reported to be effective in reducing the morbidity of HF in animal studies.²⁵ Resolving the discrepancies concerning the switch of metabolic fuel sources in hypertrophy and failure will require careful consideration of a number of complicating factors such as serum free fatty acid levels, the extent of diabetes, hormonal and catecholamine status, and the severity or stage of disease progression. At present, the prevailing view is that the stereotypical shift in metabolism towards the fetal phenotype probably occurs only during late-stage HF, and that fatty acid oxidation rates are maintained at or above normal levels during moderate HF.²⁸ This conflicts, to a certain extent, with recent studies showing that transcriptional downregulation of fatty acid oxidation and oxidative phosphorylation proteins occurs early after metabolic stress,²⁹ before overt signs of HF. It is, therefore, imperative to determine the precise mechanisms responsible for metabolic remodeling at both the early and late stages of HF, since compensatory changes in the levels of allosteric regulatory intermediates (eg, acetyl CoA, citrate, nicotinamide adenine dinucleotide [NADH]), availability of necessary cofactors (eg, carnitine, required for long-chain acyl transfer across the mitochondrial inner membrane), or anaplerotic intermediates (required for optimal Krebs cycle flux), enzyme or transporter expression levels, or post-translational switches on flux-controlling enzymes may be compensating for defects to maintain normal global energy flow.

Gene expression and proteomic analyses find that oxidative phosphorylation, fatty acid oxidation, and Krebs cycle genes (and the corresponding proteins) undergo significant quantitative changes in animal models of HF.^{10,29–32} These global findings have generally been confirmed in studies of human tissue; for example, decreased expression of metabolic genes including glucose transporter 1, glucose transporter 4, PPAR α , muscle CPT-1, medium-chain acyl-CoA dehydrogenase (MCAD), and uncoupling protein 3 paralleled a reduction in the level of the Ca²⁺-handling protein SER-CA2a in left ventricular tissues obtained from HF patients (either with or without diabetes) undergoing left ventricular assist device (LVAD) implantation.¹⁶ Interestingly, downregulation of the mRNAs associated with metabolism often occurs within days of the initiating metabolic stress²⁹ and is accompanied by alterations in signal transduction pathways, including those involved in extracellular matrix remodeling, inflammatory responses, cell growth, and apoptosis.^{29,33} The changes in metabolic gene expression are typically manifested as changes in the expressed protein levels^{30,31} with commensurate defects in function, but not in all cases. For example, in human,³⁴ rat,³⁴ mouse,³⁵ and canine¹⁰ HF, there is an early decrease in mRNA levels of MCAD, yet the levels and activity of the protein may only be decreased in the later stages of dysfunction. A consistent finding is that the mRNA and protein levels of many

subunits of complexes I to V of the electron transport chain are reduced in HF,^{29,31,33,36} whereas various enzymes of the Krebs cycle or glucose utilization can either increase or decrease.^{31,36} Most global studies of HF have not investigated the extent of PTM of the mitochondrial proteome, which we propose represents a more rapid and dynamic level of regulation beyond changes in gene expression.

The substantial effects of HF on the global network of metabolic enzymes is consistent with the idea that the activity of the master regulator of metabolism, PGC1 α , is decreased.^{33,37} PPAR α expression, and its downstream-regulated genes, decrease with advanced-stage HF,³⁸ and PGC1 α knockout mice display accelerated HF in response to aortic banding, suggesting a cause and effect relationship.^{13,39} Recent data demonstrating that a PPAR α polymorphism that impairs PPAR-mediated transcription is correlated with late-stage HF in humans also supports the hypothesis that inadequate PPAR α signaling may exacerbate cardiac decompensation.⁴⁰ Impaired signaling downstream of PGC1 α decreases mitochondrial biogenesis (via NRF1 and ERR α). It also causes reductions in the expression of genes involved in fatty acid import and oxidation (via PPAR) and the electron transport chain (ERR α , NRF1, Tfam). Concomitant upregulation of glucose utilization results from ERR α activation via a decrease in the expression of the negative regulator, pyruvate dehydrogenase kinase 4.⁴¹ Recently, a PPAR γ /Hif-1 α regulatory interaction has also been implicated in the enhancement of glycolytic gene expression associated with the hypertrophic response.⁴²

Observations that the PGC1 α pathway may be downregulated during HF have spurred investigation of the potential therapeutic use of agonists of PPAR α (fibrates) or PPAR γ (glitazones^{43–46}). Beneficial effects of fibrates, used primarily for the treatment of hyperlipidemia,⁴⁷ have been reported in several animal models of HF,^{47–50} and initial reports from clinical trials so far have indicated mixed results.^{43,51–54} The safety of glitazones, employed in diabetes therapy, has recently been called into question with the observation that several of the major PPAR γ -activating drugs are associated with increased cardiovascular morbidity and mortality.^{45,55–58} Indeed, cardiovascular and other complications linked to the use of PPAR γ agonists has led to restrictions on the use of glitazones or their withdrawal from several markets.

The discussion above highlights a certain lack of accord as to exactly which metabolic changes are the primary determinants of the transition from compensated hypertrophy to HF. Since the heart completely turns over its supply of ATP approximately every 10 seconds,²⁵ it is fundamentally obvious that a significant deficiency of energy supply in the face of continuous energy demand would very quickly lead to acute pump failure, hence the nonfailing hypertrophied heart must have adequate ATP-generating capacity.^{59,60} Nevertheless, it is likely that the reserve capacity of oxidative phosphorylation diminishes if the metabolic stress persists or demand increases. This is borne out by studies showing that myocardial oxidative capacity is operating close to its maximum in the failing heart.^{11,61} Similar limitations in metabolic reserve are evident in PPAR α -deficient animals.⁶² A decline in high-energy phosphate levels, as well as a decrease in phosphoryl transfer flux through the creatine kinase system,⁶³ correlates significantly with contractile dysfunction in late-stage HF.^{3,25} At the level of isolated mitochondria, impaired function has been reported

in pressure overload⁶⁴, rapid pacing³⁶ or microembolization-induced HF.⁶⁵ The precise nature of the mitochondrial defect is incompletely understood, but may involve an increased sensitivity of cytochrome oxidase to inhibition by nitric oxide,⁶⁶ alterations in the assembly of electron transport chain supercomplexes,⁶⁷ impaired mitochondrial biogenesis, oxidative damage, or specific PTMs.

Another key question is whether the metabolic alterations associated with HF can be reversed by therapy. The increased use of LVADs in late-stage HF patients has allowed investigators to determine whether normalization of mechanical load on the heart can reverse the genomic and proteomic alterations in metabolic pathways. Partial reversion of the transcriptome towards normal levels has been noted,⁶⁸ including genes involved in energy metabolism,⁶⁹ although this finding is not universal.⁷⁰ Whether the reverse remodeling of the mRNA profile is accompanied by restoration of enzyme levels or activity after LVAD treatment is not yet known.

Cardiac resynchronization therapy (CRT) provides another opportunity to determine whether HF therapy reverses the normal metabolic phenotype, although CRT only partially corrects abnormal mechanical loading of the failing heart. In terms of gene expression, many of the HF-associated changes in oxidative phosphorylation-related mRNA transcripts, as well as Ca²⁺ handling proteins, are reversed with CRT,^{32,71} and a recent proteomic study of canine HF indicates that changes in both the levels and PTMs of mitochondria can also be normalized.³⁶

To summarize, metabolic remodeling, and by extension mitochondrial regulation, are intimately tied to HF progression. There is consensus that mitochondrial regulation is likely to involve changes in expression of key metabolic genes. However, a recent explosion of proteomic data has revealed a host of PTMs in mitochondria including phosphorylation, S-nitrosylation, and lysine acetylation, to name only a few. The roles these modifications play in regulation of mitochondrial function are only beginning to emerge, and the extent to which they may participate in the metabolic transitions that underlie HF is uncharted territory. In the following section, we review the emergence of phosphorylation as a signaling modality, both in and around the mitochondrion, highlighting the work in cardiac systems and implications for HF.

Phosphorylation as a Mitochondrial Regulatory Strategy: Inside, Outside, and in Between

Given that the earliest evidence for an ATP-dependent protein kinase capable of phosphorylating proteins came from studies of rat liver mitochondrial extracts,⁷² it is ironic that many details of mitochondrial protein phosphorylation remain poorly characterized when compared with the sophisticated understanding of phosphorylation-dependent signaling in other cellular compartments. Elucidation of the regulation of the activity of the pyruvate dehydrogenase complex (PDC) in the late 1960s⁷³ also served as an early example of a kinase/phosphatase-dependent regulatory cascade. This complex, composed of 3 principle subunits (pyruvate dehydrogenase [E1], dihydrolipoamide acetyltransferase [E2], and dihydrolipoamide dehydrogenase [E3]), is the bridge between glycolysis and the

tricarboxylic acid cycle (TCA) cycle, catalyzing the conversion of pyruvate to acetyl CoA. It is subject to regulation both by allosteric effectors (eg, inhibition by acetyl CoA, NADH, and GTP; activation by CoA, NAD⁺, and AMP) and by phosphorylation. Phosphorylation of the E1 subunit of the complex at serines 264, 271, and 204 by pyruvate dehydrogenase kinase (PDK)^{74,75} inactivates the complex, while dephosphorylation, mediated by pyruvate dehydrogenase phosphatase, activates the complex. The phosphorylated state is enhanced by high acetyl CoA, NADH, and ATP levels and is decreased by pyruvate. Consistent with the endosymbiont theory that mitochondria in eukaryotic cells arose after engulfment of a proteobacterium, PDK shows sequence similarity to histidine kinases,^{76,77} widely distributed sensory transducers in prokaryotes.⁷⁸ Although mitochondrial PDK isoforms have apparently evolved to phosphorylate serines rather than histidines, they have little homology with other mammalian Ser/Thr kinases.⁷⁶ PDK is inhibited by dichloroacetate,⁷⁹ accounting for the activation of the complex by this compound. The pyruvate dehydrogenase phosphatase–mediating activation of the PDC requires Mg²⁺ (or Mn²⁺) as a cofactor and is stimulated by Ca²⁺.⁸⁰ It belongs to the PP2C superfamily of phosphatases⁸¹ and is a heterodimer of catalytic and regulatory subunits.

A similar atypical kinase system is involved in the regulation of branched-chain amino acid catabolism.⁸² The oxidative decarboxylation of valine, leucine, and isoleucine is carried out by the branched-chain α -ketoacid dehydrogenase (BCKD) complex. The complex contains a thiamine pyrophosphate-dependent branched-chain α -ketoacid decarboxylase (E1) that is inhibited by phosphorylation of serine 193 through BCKD kinase (BCKDK) in a mechanism analogous to that of PDC regulation. BCKDK is inhibited by the endogenous metabolite α -ketoisocaproate. BCKDK-mediated phosphorylation and inhibition of the complex occurs during protein starvation, presumably as a means to preserve branched chain amino acid levels,⁸³ and impairment of this pathway due to BCKD deficiency underlies maple syrup urine disease, characterized by severe neurological dysfunction.⁸⁴

The phosphatase responsible for dephosphorylating BCKD was recently shown to be a mitochondrially targeted member of the PPC2 family referred to as PP2Cm.⁸⁵ Phosphatase PP2Cm was first cloned by Dai and colleagues⁸⁶ and called PPC2 κ , but its mitochondrial localization was not recognized until Lu and colleagues⁸⁷ demonstrated that it was exclusively targeted to mitochondria and plays a role in modulating Ca²⁺-dependent permeability transition pore (PTP) activation and cell survival. ShRNA-mediated knockdown of PP2Cm had no effect on Ψ_m or mitochondrial oxygen consumption but caused a decrease in the threshold for activation of the mitochondrial PTP by Ca²⁺, decreased neonatal cardiomyocyte survival in culture, and increased hepatic injury and apoptosis in vivo.⁸⁷ Joshi and colleagues⁸⁸ cloned the same protein, calling it PTMP, and showed that the recombinant enzyme could dephosphorylate the BCKD complex at a higher rate than it could dephosphorylate PDC, indicating that it was distinct from the PDC phosphatases. PP2Cm was shown to bind to BCKD to dephosphorylate Ser293 in the presence of BCKD substrates, and knocking out PP2Cm in mice results in a phenotype resembling maple syrup urine disease.⁸⁵ PP2Cm has a broad tissue distribution, is particularly abundant in heart, brain, kidney, pancreas, and ovaries, and requires activation by divalent cations (Mg²⁺ or Mn²⁺).⁸⁶ Like other phosphatases of the PP2C class, it is insensitive to the protein phosphatase 1 (PP1), 2A, and 2B inhibitor okadaic acid.⁸⁶

Phosphoprotein targets of PP2Cm other than BCKD have yet to be identified but, intriguingly, knockdown of PP2Cm activates the mitogen-activated protein kinase (MAPK) stress response pathway and the levels of PP2Cm are decreased in hypertrophied and failing hearts.⁸⁷

In contrast to the two mitochondrial matrix kinase/phosphatase regulatory pathways described above, much less is known about phosphorylation-dependent regulation of the activity of other enzymes involved in mitochondrial metabolism. Indeed, for many years, the presence of kinases and phosphatases in mitochondria has been only indirectly inferred from the detection of phosphorylated sites on mitochondrial proteins. More recently, proteomic methods have provided a more detailed picture of the sites and scope of mitochondrial protein phosphorylation.^{89–94} A study of rat liver mitochondria identified 228 phosphoproteins with 447 phosphorylation sites, and a comprehensive phosphoproteome analysis of human skeletal muscle identified 77 mitochondrial phosphoproteins, including 116 phosphoserine, 23 phosphothreonine, and 16 phosphotyrosine sites.⁹³ Bioinformatic analysis of those sites identified consensus sites for protein kinase A (PKA), protein kinase C (PKC), casein kinase II, DNA-dependent kinase, and tyrosine kinase. Another recent phosphoproteome analysis of murine cardiac muscle mitochondria identified 238 phosphorylation sites on 181 phosphoproteins.⁹⁴ In addition to the expected consensus sites corresponding to targets of phosphorylation by PDK and BCKDK, consensus sequence analysis of the sites corresponded to phosphorylation by Src kinase, glycogen synthase kinase 3 (GSK-3), extracellular-signal-regulated kinase (ERK) 1/ERK2/cell division protein kinase 5, PKC, PKA, and epidermal growth factor receptor (EGFR). Clearly, phosphorylation sites abound on mitochondrial proteins and appear to be targeted by all major classes of kinases.

Questions still remain, however, about the identity and location of the kinases and phosphatases involved and whether regulation occurs acutely and reversibly in situ or during the process of import and assembly of multisubunit complexes. What is becoming clear is that proteins in the 4 mitochondrial compartments—at the outer membrane, the intermembrane space (IMS), the inner membrane, and the matrix—are likely to be regulated by phosphorylation in very specific ways.

Cyclic AMP-Dependent PKA: Tethering to the Outer Membrane

There is abundant evidence that several different families of kinases and phosphatases are docked to, or can be translocated to, the mitochondrial outer membrane. Although many questions need to be answered as to how these signals might cross the outer membrane, there is also evidence supporting modification of IMS-facing protein surfaces. Among the mitochondrial targets consistently found to be phosphorylated at the outer membrane are the apoptotic regulatory proteins (B-cell lymphoma 2 [Bcl-2],^{95,96} Bcl-2-associated death promoter [BAD],^{97,98} Bcl-2-associated X protein⁹⁹), voltage-dependent anion-selective channel proteins (VDACs) (1, 2, and 3), TOM70, and several enzymes involved in fatty acid transport or metabolism (ACS1, CPT1, and GPAT1).⁵ The impact of phosphorylation/dephosphorylation on the kinetics of most of these targets has not yet been investigated, yet circumstantial evidence would suggest that all of the major functions of the outer membrane,

ie, cell death/survival signaling, protein import, and ion, substrate, or nucleotide transport, may be regulated by the closely associated kinase/phosphatase complexes. These observations indicate that the study of local mitochondrial signaling modules may be a fertile area of investigation in the context of hypertrophy and HF.

A kinase anchoring proteins (AKAPs) constitute a major class of outer membrane scaffolding proteins. AKAPs interact with the dimerization domains of the regulatory (R) subunit of cAMP-dependent protein kinase (PKA) and are responsible for localizing the kinase to a particular target protein or cellular compartment. S-AKAP84, an AKAP expressed in male germ cells that specifically interact with the RII subunit of PKA, inserts into the mitochondrial outer membrane and can cause redistribution of a fraction of the PKA pool to the mitochondria.¹⁰⁰ Similarly, tissue-specific splice variants of the dual-specificity (capable of interacting with both RI and RII PKA isoforms) AKAP, D-AKAP1, were shown to direct the AKAP to either the ER or to mitochondria: targeting was strictly dependent on the first 30 amino acids of the N-terminus. The ability of D-AKAP1 to target PKA to the mitochondrial outer membrane plays a crucial role in regulating cell death, as it mediates the phosphorylation of the proapoptotic “BH3-domain only” protein BAD at Ser 112 in response to interleukin 3 stimulation,⁹⁸ thereby preventing its dimerization with Bcl-2 family proteins and promoting cell survival. Interestingly, AKAP121 (also known as D-AKAP1 or 1c¹⁰¹), not only localizes PKA regulatory subunits and the holoenzyme to the mitochondria, but also contains a K homology domain RNA-binding motif that interacts with a 3'UTR structural motif in the mRNAs of several mitochondrial proteins including the Fo-f subunit of mitochondrial ATP synthase and manganese superoxide dismutase (MnSOD).¹⁰² Thus, mitochondrial targeting can be facilitated through hormonal stimulation by enhancing both the local translation and trafficking of the nascent protein to the vicinity of the mitochondria. Mitochondrial AKAPs including AKAP121¹⁰³ and the peripheral benzodiazepine-associated protein, PAP7,¹⁰⁴ have also been implicated in the PKA-dependent regulation of cholesterol transport into the mitochondria during steroidogenesis.

Another AKAP, the Wiskott–Aldrich syndrome protein family member, WAVE-1, resides in a complex with PKA, PP1, BAD, and glucokinase in liver mitochondria.⁹⁷ Glucose deprivation results in the dephosphorylation of BAD and the stimulation of apoptosis, demonstrating that WAVE-1 plays a role in integrating glycolytic flux with cell death signaling. The small GTPase, Rab32, can also function as an AKAP. It possesses a 20 amino acid motif that forms a high-affinity amphipathic helical binding domain for PKA RII and is tethered to the mitochondrial outer membrane through lipid modification of two cysteines in its C-terminus.¹⁰⁵ GTP binding and hydrolysis is not required for Rab32's AKAP function, but it is required for its effects on mitochondrial dynamics.¹⁰⁵ A local PKA-mediated phosphorylation signal also appears to play a role in mitochondrial fission through another GTPase, the dynamin-related protein 1 (Drp1),^{106,107} which has been shown to be a substrate for PKA.

PTEN-Inducible Kinase

PTEN-inducible kinase (PINK1) is a kinase that contains an N-terminal mitochondrial targeting sequence and shares sequence homology with Ca²⁺/calmodulin-regulated

kinases.¹⁰⁸ Mutations in the PINK1 gene have been linked to hereditary Parkinson's disease, and defective PINK1 function is associated with oxidative stress, increased mitochondrial fission, increased mitophagy, impaired oxidative phosphorylation, and cell death, which can be mimicked in neural cell lines by PINK1 knockdown.¹⁰⁹ PINK1 is imported across the outer membrane and then cleaved by the inner membrane protease PARL while its leader sequence and N-terminal transmembrane domain are partially transferred across the inner membrane,¹¹⁰ thus releasing the kinase into the IMS. Cleavage and release of partially imported PINK1 accounts for a cytosolic fraction of PINK1, and disruption of this import/cleavage process, for example, by mitochondrial depolarization, results in PINK1-mediated recruitment of the E3 ubiquitin ligase Parkin to the mitochondria, initiating degradation.¹¹¹ Recently, it was shown that enhancing PKA targeting to mitochondria can reverse the effects of PINK1 knockdown, restoring mitochondrial function, decreasing mitochondrial fission and preventing cell death.¹¹⁰ This effect was attributed to PKA-mediated phosphorylation of Drp1. The full spectrum of direct PINK1 substrates has not been characterized, but known targets include the heat shock protein TRAP1,¹¹² mitofusin,¹¹³ HtrA2(Omi),¹¹⁴ and Parkin itself.¹¹⁵

Because PINK1 is ubiquitously expressed, it is possible that it also regulates mitochondrial function in the heart. In fact, a recent study by Billa and colleagues¹¹⁶ showed that PINK1 knockout mice develop left ventricular dysfunction and hypertrophy as early as 2 months of age. The knockout mice also displayed increased oxidative stress and impaired mitochondrial function. Notably, in the same study, PINK1 levels were also shown to be reduced in end-stage human HF.

PKA Signaling to the IMS and Mitochondrial Inner Membrane

Tethering of PKA and other kinases to the outer membrane would be expected to target protein domains facing the cytosol, adjacent organelles, or mitochondrial precursor proteins on their way through the peptide import apparatus. However, numerous phosphorylation sites on intermembrane protein domains, as well as on matrix-oriented aspects of mitochondrial proteins, have been detected.^{89–91,93} Dynamic and rapid regulation of such sites would suggest that kinases and phosphatases should be present in these compartments,¹¹⁷ but in many cases, it is not completely clear where and when the phosphorylation event may have occurred. Cyclic AMP-dependent phosphorylation of mitochondrial proteins, as well as the presence of PKA associated with isolated mitochondria, was described many years ago.¹¹⁸ Since then, the presence of PKA in the IMS or in the mitochondrial matrix has been suggested in several studies.^{119–123} However, a careful reading of the oft-cited literature indicates that the proof for a matrix-localized PKA is not unequivocal given the difficulty in distinguishing intramitochondrial from surface-tethered PKA, since the task of completely removing the outer membrane is challenging. More often than not, matrix localization of the kinase is implied by cAMP-dependent changes in the activity or phosphorylation status of inner membrane proteins. This is a logical leap of faith but does not take into account alternative explanations, such as effects on trafficking of mitochondrial subunits, changes in other signaling intermediates such as Ca²⁺ or reactive oxygen species, or that the PTM could be taking place in damaged mitochondria.

Although these caveats need to be kept in mind, there is extensive experimental support that complex I and complex IV of the respiratory chain are subject to cAMP-dependent phosphorylation. The activity of the NADH-ubiquinone dehydrogenase complex (complex I) is increased in response to the cell-permeant dibutyl-cAMP¹²⁴ and it is phosphorylated in a PKA-dependent way on multiple subunits including NDUFS4¹²⁵ B16.6 (NDUFA13), B14.5a (NDUFA7), B14.5b (NDUFC2),¹²⁶ ESSS (NDUFB11), and MWFE (NDUFA1).¹²⁷ A recent skeletal muscle phosphoproteome confirmed phosphorylation sites on NDUFB11 and added NDUFA3 and NDUFV3 as well.⁹³ Although the evidence for phosphorylation of complex I subunits is quite convincing, it still remains to be determined whether changes in activity involve a matrix- or intermembrane space-resident PKA or are effected through modulation of subunit import and assembly, as has been demonstrated for NDUFS4.^{125,128} In this latter case, the phosphorylation of the mitochondrial targeting sequence on the precursor protein stabilizes its interaction with the chaperone, HSP70, thereby facilitating the transport, processing, and retention of the mature protein within the matrix for assembly into the complex. Phosphorylation-dependent mitochondrial targeting of several other proteins has also been shown^{129,130} and, as mentioned above, AKAPs may be involved in the cotranslational shepherding of mRNA and protein to the mitochondrial compartment.

Ambiguity also exists with respect to the regulation of cytochrome oxidase (complex IV) by cAMP-dependent phosphorylation. Serine, threonine, and tyrosine phosphorylation on multiple subunits (including I, II, IVi1, Vb) of cytochrome oxidase has been reported^{131,132} and PKA-dependent phosphorylation is postulated to underlie inhibition of its activity at high ATP/adenosine diphosphate (ADP) ratios; an effect countered by Ca²⁺-mediated dephosphorylation.^{133,134} A direct interaction between cytochrome oxidase and a PKA/RI-GST fusion protein in vitro has also been reported.¹²⁰ Again, the question remains as to whether phosphorylation-dependent changes in activity are related to phosphorylation/dephosphorylation events occurring on already-assembled complexes or are related to dynamic trafficking or assembly events. With regard to the latter, Rosca and colleagues⁶⁷ have recently reported increased levels of threonine (but unchanged serine) phosphorylation of complex IV subunits that were *not* incorporated into respiratory chain supercomplexes (I +III+IV) in mitochondria isolated from a canine microembolization-induced HF model, while phosphoserine levels within the intact supercomplex were decreased.¹³⁵ In this HF model, alterations in the levels of supercomplexes were associated with defects in respiratory flux supported by both complex I (glutamate) and complex II (succinate) substrates. Moreover, a reduced respiration directly supported by tetramethyl-p-phenylenediamine/ascorbate was also observed, indicating a defect in cytochrome c oxidase itself.

Assuming that there is dynamic regulation of PKA-modulated sites facing the mitochondrial matrix, we must next ask: what is the source of cAMP that regulates the kinase? In 2003, Zippin and colleagues¹³⁶ reported that the “soluble adenylate cyclase” (sAC), originally isolated from testis, was broadly distributed across many tissues and cell lines and was also enriched in nuclear and mitochondrial fractions, as well as at microtubule organizing centers. More recently, Acin-Perez and colleagues¹³⁷ explored the mitochondrial role of sAC in more detail. sAC is a receptor/G protein-independent cyclase that is modulated by

bicarbonate and the authors showed that bicarbonate could activate cAMP production in mitochondria when it was either added exogenously or derived from carbonic anhydrase-mediated conversion of carbon dioxide produced by the TCA cycle. The same group also provided evidence for an intrinsic mitochondrial phosphodiesterase,¹³⁸ completing the characterization of the mitochondrial cAMP signaling pathway. Notably, the effects of activating this cAMP-dependent pathway in either HEK cells or in isolated mouse liver mitochondria were to increase complex IV activity and mitochondrial respiration¹³⁷—the opposite of the inhibitory effect in bovine heart mitochondria¹³³ or macrophages¹³¹ discussed above. This suggests that the activity of the cytochrome c oxidase complex may be either upregulated or downregulated by phosphorylation at several different sites, perhaps by several different kinases.

Cyclic GMP-Dependent Protein Kinase G

When compared with cAMP-dependent regulation of oxidative phosphorylation, there is much less evidence to support intramitochondrial functional regulation by cyclic guanosine monophosphate (cGMP). Nevertheless, the nitric oxide (NO)/cGMP axis has been linked to the activation of mitochondrial biogenesis¹³⁹ and protein kinase G (PKG)-mediated signaling to the mitochondria is known to play a crucial role in modulating the protective effect of cardiac preconditioning,^{140–142} possibly through recruitment of signaling components (signalosomes) to the mitochondria.¹⁴³ All of the steps of this “outside in” signaling modality have not been worked out in detail, but GSK-3 β has been shown to be an important point of convergence for many preconditioning stimuli, including PKG-linked pathways, with GSK-3 β inhibition strongly correlating with protection.¹⁴⁴ The downstream mitochondrial targets of PKG and/or GSK-3 β are just beginning to be worked out, but a recent report by Das and colleagues¹⁴⁵ identified GSK-3 β -mediated VDAC2 phosphorylation as one target. VDAC2 dephosphorylation in the presence of a GSK-3 β inhibitor was shown to be correlated with a decrease in ATP uptake and hydrolysis by chemically uncoupled mitochondria, which could theoretically be an energy-sparing mechanism during ischemia/-reperfusion. In skeletal muscle mitochondria, 8 phosphorylation sites were identified on VDAC1 (7 Ser, 1 Tyr), 7 were found on VDAC2 (5 Ser, 2 Thr), and 5 on VDAC3 (1 Ser, 3 Thr, 1 Tyr), but the kinases involved, and their possible function effects, have yet to be investigated.

Early studies on the distribution of guanylate cyclase (GC) in heart and brain indicated that, in addition to the soluble GC fraction, there is also GC in the particulate fraction (containing mitochondria and synaptosomes).^{146,147} Subsequently, Sessa and colleagues¹⁴⁸ reported that NO donors (S-nitroso-N-acetyl-penicillamine, sodium nitroprusside) increased cGMP in a protein fraction obtained from highly purified mitochondria, and that membrane-permeable cGMP analogs, but not cGMP itself, could evoke cytochrome c release from intact mitochondria. This effect was inhibited by ODQ, an NO-dependent GC inhibitor, but was not accompanied by loss of membrane potential or swelling, indicating that the MPT was not involved. The mechanism of the NO/cGMP-induced outer membrane permeabilization and cytochrome c release was not determined, nor was the possible involvement of PKG investigated; however, the effect was linked to the induction of apoptosis in adult cardiomyocytes.¹⁴⁸ This negative effect on cell survival stands in contradistinction to the

protective effect of NO/cGMP discussed above, highlighting the need to further investigate the potential for multiple downstream effects of NO-related PTMs on mitochondria.

Protein Kinase C

Mitochondria are also a nodal point for PKC action, and early studies indicated that PKC could phosphorylate a number of proteins in isolated mitochondria.¹⁴⁹ Specific PKC isoforms can migrate to mitochondria upon activation, most likely by interacting with receptors for activated/inactive C-kinase (RACK/-RICK)^{150,151} present on the outer membrane. PICK1 is one such PKC-interacting protein, and in NIH 3T3 cells, it was shown to be localized to mitochondria.¹⁵² Upon serum stimulation, mitochondria, along with PICK1, translocated to the perinuclear area of the cell and facilitated PKC α localization to the same region. In contrast, phorbol ester activation resulted in PKC α localization to the plasma membrane without affecting PICK1 localization. PICK1 mitochondrial localization could be eliminated by deletion of its PDZ domain, while deletion of a C-terminal PICK1 interaction domain (QSAV) on PKC α disrupted its mitochondrial localization after serum stimulation. Thus, PICK1 appears to be a resident PKC α docking site on mitochondria that does not depend on prior PKC activation. PICK1 interaction with PKC α was shown to be involved in the mechanism of protection against etoposide-induced apoptosis, which was associated with the phosphorylation of Bcl-2.¹⁵³

The role of PKC in the modulation of mitochondrial function has been most extensively studied in the context of ischemia-reperfusion injury.^{154–156} In this case, rather than involving a mitochondrially resident PKC, specific isoforms appear to be translocated to mitochondria in response to preconditioning stimuli. Overexpression of constitutively active PKC ϵ in mice is cardioprotective¹⁵⁴ and PKC ϵ and PKC η appear in the particulate fraction of rabbit heart lysates in proportion to the number of short occlusion/reperfusion cycles to which the heart is subjected.¹⁵⁷ PKC ϵ activation also leads to PKC δ translocation to mitochondria, and the interplay between these kinases. Modification of the metabolic proteins is thought to confer a protected phenotype,¹⁵⁸ although other observations, based on isoform-specific peptide inhibitors/activators, suggest that PKC ϵ and PKC δ have opposite effects on ischemic damage, ie, protective in the case of PKC ϵ and damaging in the case of PKC δ .^{159,160}

Baines and colleagues¹⁶¹ showed that several mitochondrial proteins could be detected after immunoprecipitation with an anti-PKC ϵ antibody. Among these, VDAC1 was shown to be phosphorylated by PKC ϵ in vitro; moreover, exogenously added PKC ϵ could suppress Ca²⁺-evoked PTP activation in isolated mitochondria. The threshold for PTP activation was increased in mitochondria from PKC ϵ -overexpressing mice and lowered in mice expressing a catalytically inactive PKC ϵ . The PKC ϵ signaling complex involved in cardioprotection was also shown to contain ERK, JNK, and p38 MAPK, and the activation of the complex correlated with phosphorylation and inactivation of the proapoptotic protein BAD.¹⁶² The mechanism of how PKC ϵ and its signaling complex may be translocated to the mitochondria is still unclear; however, a recent study by Budas and colleagues¹⁶³ demonstrated that Hsp90 plays a role in the import of PKC ϵ across the outer membrane, facilitating the phosphorylation of aldehyde dehydrogenase 2 at the inner membrane. Whether this is a

common mechanism for other kinases to be shuttled into the mitochondria is not known. A mitochondrial RACK for PKC ϵ has not been identified; however, peptide inhibitors of the PKC-RACK interaction abolish ischemic preconditioning.¹⁵⁹ In addition, Ogbi and Johnson¹⁶⁴ reported that membrane-permeant peptide inhibitors of PKC ϵ translocation abrogated the hypoxia-induced phosphorylation of an 18kDa mitochondrial target (hypothesized to be a component of cytochrome c oxidase) and also found a direct interaction between PKC ϵ and cytochrome c oxidase.

In a recent proteomic study of murine cardiac mitochondria, more than 40 phosphoproteins containing sites compatible with a PKC consensus sequence were identified.⁹⁴ These included a variety of proteins such as subunits of PDK, succinyl CoA ligase, mitochondrial creatine kinase, A-kinase anchor protein (AKAP) 1, Bcl-2-like 13 protein, among others. Interestingly enough, apart from mitochondrial creatine kinase, another recent study in skeletal muscle⁹³ reported a completely different set of putative PKC-regulated mitochondrial phosphoproteins, including apoptosis-inducing factor 1, ATPase inhibitory factor 1, carnitine O-palmitoyltransferase 1, NADH-cytochrome b5 reductase, aspartate aminotransferase, mitochondrial inner membrane protein, prohibitin-2, prostaglandin E synthase 2, ADP/ATP translocase 3, cytochrome b-c1 complex Rieske subunit, and VDACs 1, 2, and 3. This lack of agreement regarding potential mitochondrial PKC targets, even between two striated muscle types, will make it challenging to narrow down the most important targets of PKC involved in cell protection, physiological regulation, or pathological effects.

The relevance of potential mitochondrial PKC targets to the progression of HF has not been clearly elucidated. However, selective activation of either PKC ϵ or PKC δ has the same effect on cardiac growth, inducing a nonpathologic hypertrophic response.¹⁵⁹ Chronic activation of PKC ϵ in transgenic mice induces compensatory hypertrophy with age.¹⁶⁵ In the hypertrophic G α_q -overexpressing mouse model, PKC ϵ activation mitigates cardiomyopathy and increases the localization of this kinase to the particulate fraction, while inhibition of PKC ϵ leads to a lethal dilated cardiomyopathy phenotype with less kinase present in the particulate fraction.¹⁶⁶ It remains to be determined whether alterations in the mitochondrial phosphoproteome underlie the functional outcomes in these models.

Mitochondrial Tyrosine Kinases and Phosphatases

Tyrosine phosphorylation of mitochondrial proteins was recognized 25 years ago,¹⁶⁷ when it was thought to be primarily localized to the outer membrane. Since then, a number of protein tyrosine kinases (Src, Lyn, Fyn, Csk, Fgr, Abl, EGFR) and phosphatases (SHP-2, MKP-1, PTP1D, PTPMT1) have been shown to be either docked to mitochondria as part of signaling modules¹⁵⁶ or localized within mitochondria.^{168,169} The nonreceptor protein tyrosine kinase c-Src has been demonstrated to play a role in the regulation of oxidative phosphorylation at several sites. Miyazaki and colleagues¹⁷⁰ showed that in osteoclasts, Src-mediated phosphorylation of cytochrome c oxidase subunit II increases complex IV activity and modulates the rate of bone resorption. Cytochrome c itself was also shown to be a target of tyrosine phosphorylation in the heart at a site near its membrane-tethering cardiolipin-binding domain.¹⁷¹ More recently, Feng and colleagues¹⁷² demonstrated that

phosphorylation of tyrosine residues (Y190, 194) in the IMS-facing cavity of the adenine nucleotide translocase (ANT) is vital for ADP/ATP exchange. Src or Lck were capable of phosphorylating the ANT sites in vitro, and an inhibitor of Src-kinase could block phosphorylation in cells. Furthermore, these authors also showed that dephosphorylation of Y194 occurs during isoflurane-induced cardiac preconditioning in rat hearts and that Src-kinase inhibition prevented ANT dephosphorylation as well as the protection against ischemia. In addition to ANT and cytochrome c oxidase, other targets of tyrosine kinase include subunits of complex I (NDUFB11, NDUFB7), complex III (cytochrome b), α -ketoglutarate/malate carrier (SLC25A11), mitochondrial inner membrane protein, VDACS 1 and 3, monoamine oxidase A, and mitochondrial creatine kinase, identified in skeletal muscle mitochondria,⁹³ while in cardiac muscle mitochondria, the ANT phosphosite and several additional phosphotyrosine-containing proteins were identified, including TCA cycle enzymes and mitochondrial arginase.⁹⁴

Interestingly, the aforementioned AKAP121 also plays a role in tyrosine kinase/phosphatase targeting to mitochondria. Livigni and coworkers¹⁷³ found that AKAP121 not only targets PKA to the mitochondria but also Src, through interaction with the protein tyrosine phosphatase (PTP) PTPD1. AKAP121-mediated targeting of the signaling complex was correlated with increased cytochrome c oxidase activity and enhanced ATP synthesis and the effect was PKA- and Src-dependent.

In addition to the PTPD1 targeting mentioned above, several other groups have reported PTPs in the mitochondria. PTPM1¹⁷⁴ was the first PTP shown to be localized to the matrix face of the mitochondrial inner membrane by means of a canonical N-terminal import sequence. Knockdown of PTPM1 in an insulinoma cell line enhanced ATP production and insulin secretion. Mitochondrial localization of Shp-2 and PTP1B has also been demonstrated; however, this localization was tissue specific—these phosphatases were detected in brain mitochondria but could not be detected in mitochondria from the heart, skeletal muscle, liver, or kidney.^{175–177} In accord with this observation, changes in tyrosine phosphorylation of mitochondrial proteins were observed only in brain mitochondria, and not other tissues, after treatment with ATP, a Src kinase inhibitor, or the nonspecific phosphatase inhibitor orthovanadate, and functional effects of tyrosine phosphorylation on electron transport complexes were also exclusive to brain mitochondria.¹⁷⁵

Taking the available evidence into account, it appears that there is strong evidence that cardiac mitochondrial proteins contain numerous phosphotyrosine residues, yet their impact on function is not well understood. Moreover, it remains to be proven whether rapid and reversible regulation by intramitochondrial tyrosine kinases/phosphatases occurs in the heart and, if present, under which conditions it may be altered.

MAPK Pathway–Related Kinases

The MAPK pathway typically links plasma membrane receptor activation (such as G-protein–coupled receptors or receptor tyrosine kinases such as EGFR) to a nuclear transcriptional response through a sequentially activated 3-tiered kinase cascade involving MAPK kinase (eg, RAF, activated by the G-protein RAS), MAPK kinase (MEKs), and

MAPK (ERKs, JNK, p38). As mentioned above, elements of this pathway are found in the PKC signaling modules associated with cardioprotection, and peripheral mitochondrial localization of several of these kinases has been reported.^{95,178,179} Intriguingly, there is also evidence that the EGFR receptor itself may migrate to the mitochondria upon activation and confer resistance to apoptosis in cancer cells, possibly through a direct interaction with cytochrome c oxidase subunit II,^{180,181} although immunoelectron microscopy suggests that the coassociation of EGFR with mitochondria probably occurs mainly in damaged mitochondria located within autophagosomes.¹⁸²

The MAPK pathway has been strongly implicated in modulating cell death, although depending on cell type and conditions, the effect can be either protective or death-inducing.¹⁷⁹ These effects are likely to be mediated in large part by phosphorylation of BH3-containing proteins by MAPKs. The antiapoptotic protein Bcl-2 is phosphorylated by ERK in tumor cell lines, and the phosphorylated form has a diminished ability to localize to the mitochondria.⁹⁶ The dephosphorylation of Bcl-2 was attributed to PP2A, which enhanced Bcl-2's mitochondrial localization and its antiapoptotic effects. N-terminal splice variants of PP2A have been shown to confer mitochondrial targeting to this phosphatase in neurons.¹⁸³ Bcl-2 was also shown to target Raf-1 to mitochondria, resulting in the phosphorylation of BAD and inhibition of apoptosis.⁹⁵

The MAPK pathway has been shown to modulate the cardiac hypertrophic program^{184,185} in specific ways depending on which branch of the pathway is activated. For example, activation of ERK1/2 induces a concentric hypertrophy (similar to pressure-overload hypertrophy) while MEK5/ERK5 activation induces eccentric hypertrophy (similar to volume overload).¹⁸⁴ The MAPK activation state was shown to change during hypertrophy and HF in a biphasic manner—following acute mechanical stretch or chronic pressure overload of the heart, ERK1/2, p38 kinase, Src, p90RSK, and big MAPK (BMK1) are activated¹⁸⁶ but with the transition to HF, the activity of the latter two kinases decreases. Moreover, stretch-induced activation of ERK1/2 and p38 MAPK can be blunted by PKC inhibition. At present, however, there is no information available about potential localization of these kinases to the mitochondria during compensated or decompensated hypertrophy and whether mitochondrial MAPKs influence metabolism, cell death, or redox balance in HF.

Mitochondrial ATP Synthase Complex: Case Study of the Impact of Multisite Phosphorylation

The mitochondrial ATP synthase complex has been shown by several groups to be phosphorylated on multiple subunits, including α , γ , δ , ϵ , 4, OSCP, c and g,^{89,91} β ,^{89,91} and more recently on subunits b, d, 6, and IF1⁹³ as well. Most of the reported sites are phosphoserines or phosphothreonines, but Ko and colleagues¹⁸⁷ reported tyrosine phosphorylation on the single tyrosine in the δ subunit, mediated by platelet-derived growth factor, presumably targeted to the mitochondria and activated by lysophosphatidic acid. Arrell and colleagues¹⁸⁸ identified 5 novel phosphorylation sites on the ATP synthase β subunit (S106/T107, T262, T263, T312, and T368) that were induced by cardiac preconditioning, and later Kane and colleagues¹⁸⁹ explored the impact of 4 of these sites on subunit assembly and ATPase activity by generating mutations at the equivalent residues in

the yeast β subunit. The phosphomimetic mutant T262E (mammalian T312E) completely abolished ATPase activity and led to a change in the levels of free F1, but not the intact F1Fo complex, indicating an additional effect on assembly. There was no effect of ablating phosphorylation at that site by alanine substitution. Either alanine or glutamate substitution at T58(mamm. S106) or T318(mamm. T368) decreased ATPase activity compared with the WT protein and the T58A mutation increased dimerization of the holoenzyme complex.¹⁸⁹ The other modified residues had no observable effects on function; a clear demonstration that not all phosphorylatable residues should be expected to have an impact on oxidative phosphorylation.

In addition to recent evidence showing that oxidative PTMs on the ATP synthase α subunit, including disulfide bond formation, glutathionylation, and nitrosylation are altered during the development of dyssynchronous HF and are partially reversed by cardiac resynchronization therapy (CRT),¹⁹⁰ the phosphorylation site analogous to the T318 site on the ATP synthase β subunit discussed above was phosphorylated more in the diastolic heart failure group and the complex was partially degraded.³⁶ The quantity of the ATP synthase holoenzyme was increased approximately 2-fold with CRT and its ATPase activity increased approximately 20%. The change in the phosphorylation status of the ATP synthase, along with other quantitative changes in electron transport chain complexes, TCA cycle enzymes, and fatty acid pathways, could account for improved mitochondrial function after resynchronization therapy.³⁶ The findings warrant a more detailed analysis of the complete mitochondrial phosphoproteome in this model, as well as in human cardiac tissues.

Conclusions

Novel mass spectrometry-based methods have dramatically increased the number of identified PTMs on mitochondrial proteins and it is becoming increasingly evident that, as in other cellular compartments, phosphorylation is likely to play a regulatory role. At present, except for a few individual examples, several aspects of mitochondrial protein phosphorylation are still unclear, including: (1) the fraction of phosphorylated vs dephosphorylated protein for each target; (2) the pattern, stoichiometry, and turnover of specific phosphorylation sites; (3) whether the sites are phosphorylated in situ after the protein has been imported and assembled or are modified during the assembly process; (4) which kinases and phosphatases are responsible for acute regulation and how these are targeted, imported, and retained in the IMS or matrix; and, most importantly, (5) the functional role, or lack thereof, of specific phosphorylation sites with respect to metabolic flux control.

The role played by mitochondrial phosphorylation pathways on the pathophysiology of cardiac hypertrophy and failure is just beginning to be explored; however, there are hints that the effects may be transduced at several levels. At the outer membrane, localized mitochondrial signaling modules regulate transport, cell death effectors, and mitochondrial fission/fusion. In the IMS, there is evidence for modulation of free-radical balance, nucleotide transport, respiratory chain activity, and complex assembly, while in the matrix, phosphorylation regulates enzymes involved in the TCA and amino acid metabolism, and probably also regulates electron transport chain activity and ATP synthesis. With increasing

recognition that defective mitochondrial function contributes to cardiac decompensation, the impact of phosphorylation as a mitochondrial PTM is an area ripe for further investigation.

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Figure.

Mitochondrial protein phosphorylation. Phosphorylation sites (red dots) have been identified on proteins in the 4 major compartments of the mitochondria, the mitochondrial outer membrane (MOM), intermembrane space (IMS), mitochondrial inner membrane (MIM), and the matrix, affecting the major functions of each compartment. At the MOM, A-kinase anchoring proteins (AKAPs) modulate protein translation, chaperone (Hsp70, TRAP1) interaction, precursor import and assembly, and mitochondrial BH3-protein interaction (Bad, Bcl-2) in concert with localized protein phosphatase 2A (PP2A). MOM transport processes for nucleotides, fatty acids, steroid precursors, and other substrates are also key targets of kinase action. PKC α is tethered to the MOM by PICK1, while other PKC isoforms are translocated to the MOM as part of multicomponent signaling modules activated by stress or plasma membrane receptor activation to confer protection through incompletely defined targets. PTEN-inducible kinase (PINK1) is an IMS-resident kinase (a target of PARL-mediated cleavage) that is thought to play an important role in neural cell death and has recently been linked to cardiac hypertrophy. Multiple IMS-facing phosphorylation sites on mitochondrial electron transport chain complexes (I–V) and the adenine nucleotide translocase (ANT) have been implicated in acute functional regulation of oxidative phosphorylation. Similarly, the kinases responsible for matrix-localized phosphorylation of Krebs cycle enzymes and respiratory chain complexes are unknown, except for the well-studied kinase/phosphatase cascades that regulate the pyruvate dehydrogenase (PDH) and the branched-chain α -ketoacid dehydrogenase (BCKDH) complexes. Pyruvate dehydrogenase phosphatase (PDP), protein phosphatase 2Cm (PP2Cm), and protein tyrosine phosphatase are 3 phosphatases that are almost exclusively localized to the mitochondrial matrix. Soluble adenylylase (sAC) is activated by bicarbonate, which is produced by the action of carbonic anhydrase from carbon dioxide generated by the Krebs cycle. SDH

indicates succinate dehydrogenase; MDH, malate dehydrogenase; CS, citrate synthase; IDH, isocitrate dehydrogenase; α -KGDH, α -ketoglutarate dehydrogenase; AAT, aspartate aminotransferase; PIC, inorganic phosphate carrier; VDAC, voltage-dependent anion channel; CKm, creatine kinase; CPT1, carnitine palmitoyltransferase; TOM, translocase of the outer membrane; TIM, translocase of the inner membrane.