AHA Scientific Statement

Mitochondrial Function, Biology, and Role in Disease
A Scientific Statement From the American Heart Association

Elizabeth Murphy, PhD, Chair*; Hossein Ardehali, MD, PhD; Robert S. Balaban, PhD*; Fabio DiLisa, MD; Gerald W. Dorn II, MD; Richard N. Kitsis, MD; Kinya Otsu, MD, PhD; Peipei Ping, PhD; Rosario Rizzuto, MD; Michael N. Sack, MD, PhD*; Douglas Wallace, PhD; Richard J. Youle, PhD†; on behalf of the American Heart Association Council on Basic Cardiovascular Sciences, Council on Clinical Cardiology, and Council on Functional Genomics and Translational Biology

Abstract—Cardiovascular disease is a major leading cause of morbidity and mortality in the United States and elsewhere. Alterations in mitochondrial function are increasingly being recognized as a contributing factor in myocardial infarction and in patients presenting with cardiomyopathy. Recent understanding of the complex interaction of the mitochondria in regulating metabolism and cell death can provide novel insight and therapeutic targets. The purpose of this statement is to better define the potential role of mitochondria in the genesis of cardiovascular disease such as ischemia and heart failure. To accomplish this, we will define the key mitochondrial processes that play a role in cardiovascular disease that are potential targets for novel therapeutic interventions. This is an exciting time in mitochondrial research. The past decade has provided novel insight into the role of mitochondria function and their importance in complex diseases. This statement will define the key roles that mitochondria play in cardiovascular physiology and disease and provide insight into how mitochondrial defects can contribute to cardiovascular disease; it will also discuss potential biomarkers of mitochondrial disease and suggest potential novel therapeutic approaches. **(*Circ Res.* 2016;118:1960-1991. DOI: 10.1161/RES.0000000000000104.)**

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The mitochondria are recognized as a key player in cardiomyocyte cell death after myocardial infarction and cardiomyopathies. Alterations in mitochondrial function are increasingly recognized in cardiovascular disease. Although it has been suggested that the failing heart is energy starved,[1] the recent understanding of the complex interaction of the mitochondria in regulating metabolism and cell death provides novel insight and therapeutic targets. This bioenergetics perspective of cardiomyopathy can be understood as one manifestation of an array of different common clinical phenotypes, including myopathies, neuropathies, nephropathies, endocrine disorders and metabolic diseases, aging, and cancer. This is because the organs that are affected in the common "complex" diseases are the same organs that have the highest reliance on mitochondrial function.[2]

The purpose of this statement is to better define the potential role of mitochondria in the genesis of cardiovascular disease such as ischemia and heart failure (Figure 1). To accomplish this, we will define the key mitochondrial processes that play a role in cardiovascular disease, which are potential targets for novel therapeutic interventions. This is an exciting time in mitochondrial research. The past decade

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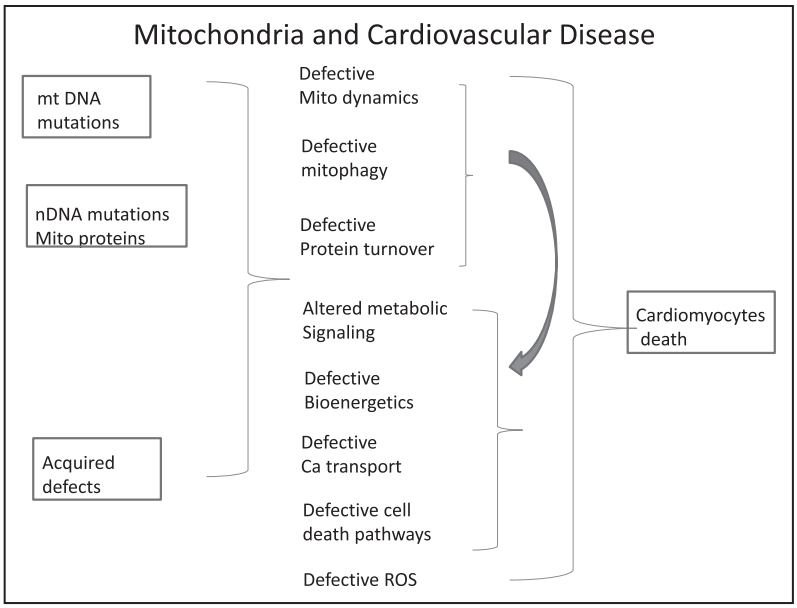

Figure 1. Mutations in mitochondrial proteins (either from mutation in mitochondrial DNA [mtDNA] or nuclear DNA [nDNA]) or acquired defects can lead to defects in mitochondrial quality control, which leads to a vicious cycle of more acquired mitochondrial defects and defects in metabolic signaling, bioenergetics, calcium transport, reactive oxygen species (ROS) generation, and activation of cell death pathways. This leads to a vicious feed-forward cycle that leads to cardiomyocyte cell death.

has provided novel insight into the role of mitochondria function and their importance in complex diseases. In the section on Mitochondrial Function, this statement will define the key roles that mitochondria play in cardiovascular physiology and disease. The section on Mitochondria and Cardiovascular Disease will provide insight into how mitochondrial defects can contribute to cardiovascular disease and will also discuss potential biomarkers of mitochondrial disease and suggest potential novel therapeutic approaches.

Mitochondria are well known as the powerhouse of the cell, and as discussed in the section on Generation of ATP: Bioenergetics and Metabolism, in an active tissue such as heart, they are responsible for generating most of the ATP in the cell. The role of posttranslational modifications (PTMs) in the regulation of metabolism is also discussed (Regulation of Function and Metabolism—The Role of PTMs). It has long been known that in addition to generating ATP, the mitochondrial electron transport chain (ETC) is also important in regulation of mitochondrial calcium. The recent identification of the proteins involved in regulation of mitochondrial matrix calcium is providing new insights into the regulation and role of mitochondrial calcium (Calcium Transport). As discussed in the section on Mitochondria and Cell Death, mitochondria are also key regulators of cell death. In the process of electron transport to generate ATP, mitochondria can be a major source of reactive oxygen species (ROS) that can both contribute to cell death and serve as a signaling molecule (Generation of ROS). Because the generation of ROS can lead to damage to mitochondrial DNA (mtDNA) and proteins, it is important for the mitochondria to have mechanisms to ensure quality control (Mitochondrial Quality Control). Quality control can occur by fission/fusion to allow segregation of damaged mitochondria (Fission/Fusion/Mitochondrial Dynamics), mitophagy to remove damaged mitochondria (Mitophagy and Mitochondrial Autophagy: Mechanisms, Overlap, and Distinctions), and ultimately cell death if the damage is too severe (Mitochondria and Cell Death). Although mitophagy is important for quality control and for removal of damaged mitochondria, based on measurement of mitochondrial protein turnover (Protein Turnover Independent of Mitophagy) it appears that mitochondrial proteins turn over at different rates, which suggests that under normal circumstances, mitophagy is not the main driver of mitochondrial protein turnover. In fact, it is suggested that the dynamics of protein turnover can provide an assessment of the physiological state. Alterations in these mitochondrial functions are important in many cardiac diseases, as discussed in the section on Mitochondria and Cardiovascular Disease. The section on Mitochondrial Myopathies: Mitochondrial Pathogenesis of Cardiomyopathy examines the mitochondrial pathogenesis of cardiomyopathy, and the section on Cardiotoxicity discusses the role of mitochondria in cardiotoxicity. The section on Biomarkers discusses potential biomarkers for mitochondrial diseases. Taken together, this American Heart Association scientific statement provides a state-of-the-art assessment of the current status of basic mitochondrial biology and how alterations in mitochondria can be major contributors to complex cardiovascular diseases.

Mitochondrial Function

Generation of ATP: Bioenergetics and Metabolism

Energy Demands of the Heart

The incessant energy requirements of the heart are sustained by the consumption of a mass of ATP daily that surpasses cardiac weight itself by approximately 5 to 10 fold.[3] This perpetual demand for energy reflects the continuous contractile functioning of the heart to sustain systemic circulation and nutrient supply. This high-energy flux translates into the cardiomyocyte having a mitochondrial volume between 23% and 32% of myocellular volume.[4] Interestingly, cardiac mitochondrial density increases from human to mouse in parallel with increasing heart rate and oxygen consumption.[4] On the basis of the role of mitochondria in energy transduction, it is not surprising that any perturbations in mitochondrial energy balance, production, or propagation would result in the

development of cardiac pathology or susceptibility to injury (Mitochondrial Myopathies: Mitochondrial Pathogenesis of Cardiomyopathy; Cardiotoxicity); however, a linear or direct correlation between mitochondrial energy metabolism and heart pathology is not clear cut. In this section, we give a brief overview of metabolism and perturbations and their consequences on myocardial function.

ETC Biology: Energetics and ROS

The final common pathway for oxidative metabolism, which generates the bulk of cardiac ATP, is the sequential passage of electrons from high (NADH or $FADH_2$) to low (molecular oxygen) redox potentials down the ETC (complexes I through IV). This stepwise electron transfer results in the active pumping of hydrogen ions out of the mitochondrial matrix into the intermembranous space. The ensuing electrochemical gradient generated across the inner mitochondrial membrane (IMM) facilitates the translocation of protons from the intermembranous space through the F_o/F_1 ATPase (ATP synthase) back into the mitochondrial matrix. This proton translocation is coupled to the phosphorylation of ADP to generate ATP. Collectively, these reactions constitute oxidative phosphorylation, and the direct synthesis of ATP from electron transfer encapsulates coupled respiration.[5] Being cognizant of the high-energy demands of the heart, it is not surprising that mutations in genes that encode for ETC proteins are linked to the development of cardiomyopathy (Mitochondrial Myopathies: Mitochondrial Pathogenesis of Cardiomyopathy).[6–8] However, it should also be recognized that dysfunction in the ETC not only affects ATP production but can concordantly impair intracellular Ca^{2+} flux (Calcium Transport), increase the generation of ROS (Generation of ROS), and alter redox balance by altering the NAD^+/NADH ratio.[9,10]

Fuel Substrates and Cardiac Energetics in Health and Disease

Mitochondrial fatty acid β-oxidation (FAO) is the most efficient and predominant substrate for energy production in the normal adult human heart, with glucose oxidation, glycolysis, lactate, and ketones additionally contributing to myocardial ATP production.[11] Regulation of cardiac energy metabolism is complex and determined by the summation of intracellular substrate concentrations, transcriptional rates and activity of metabolic enzymes, and metabolic demands of the myocardium.[12,13] However, as the heart remodels in response to hypertrophy and ischemia, marked changes in cardiomyocyte substrate metabolism can occur, with an ultimate effect on ATP levels in the decompensating heart.[14–16] The relative contribution of fatty acids diminishes with enhanced reliance on glucose utilization during the development of cardiac hypertrophy. The regulatory programs that attenuate fat utilization have been investigated extensively and include regulation at the transcriptional and posttranscriptional levels.[17–20] Moreover, this reduction involves coordinate downregulation of proteins that control fatty acid uptake by the heart and mitochondria, as well as of the enzymes that control mitochondrial FAO (Protein Turnover Independent of Mitophagy).[17,21–24] Enzymes involved in glycolytic pathways are upregulated even during early stages of cardiac dysfunction in response to increased adrenergic signaling, upregulation of fetal gene programs, and hypoxia.[25,26] The shift toward glucose metabolism improves myocardial contractile efficiency by increasing the stoichiometric ratio of ATP production to oxygen consumption in addition to minimizing oxidative losses through the mitochondrial respiratory chain uncoupling associated with free fatty acid metabolism.[27,28] Abnormally high myocardial dependence on fatty acid metabolism, as seen during ischemia or high adrenergic states, increases cardiac oxygen consumption by 30% to 50%, adjusted for equivalent stroke work indexes.[29,30] Strategies to increase glucose oxidation and decrease fatty acid metabolism can improve myocardial energy efficiency by up to 30%.[31] Although the initial shift toward glucose metabolism at progressively more advanced stages of cardiac dysfunction is physiologically adaptive, the magnitude and impact of this adaption can be significantly limited by extracardiac factors, specifically the development of insulin resistance. Whole-body insulin resistance can affect cardiac energy metabolism, even in structurally normal hearts. Patients with type 2 diabetes mellitus who have otherwise normal cardiac function regenerate phosphocreatine at a significantly lower rate after exercise than control subjects without diabetes mellitus.[32] In patients with cardiomyopathy, the development of insulin resistance is linked to increased sympathetic signaling, which leads to liberation of free fatty acids from adipose tissue into the bloodstream.[33] Thus, in the failing myocardium, decreases in insulin sensitivity can lead to further reductions in glucose oxidation and deteriorations in cardiac function by depriving the heart of access to a more metabolically efficient substrate.

Tricarboxylic Acid Biology and Anaplerosis

The capacity to use multiple substrates and the plasticity to switch substrate utilization enables continuous cardiac work under a wide variety of biological and pathological circumstances. Interestingly, under some pathological conditions such as severe hypertrophy, the coupling of glycolysis and pyruvate oxidation becomes disrupted, with an increase in glucose oxidation that is insufficient to completely compensate for the reduced fat oxidation.[24,34] These perturbations in substrate partitioning and selection can become associated with reduced contractile reserve and increased susceptibility to ischemia-reperfusion injury.[34–36] Partial compensation for this energy-substrate oxidation deficit has recently been identified to occur via recruitment of alternative intermediary pathways (anaplerosis) to enhance flux through the tricarboxylic acid cycle.[23] The need for anaplerosis in the heart is well established, in that the mechanical performance of isolated rat hearts when exclusive precursors of acetyl coenzyme A (CoA) are used as substrate shows progressive deterioration with rapid restoration after introduction of anaplerotic substrates.[37] Whether the disruption of anaplerosis plays a significant role in cardiac maladaptation and whether it could be a therapeutic target for therapy are currently unknown.[27]

Metabolic Modulation as a Strategy for Cardiac Muscle Pathology

Despite the findings of altered metabolism and energetic capacity in experimental models and in patients with cardiac muscle injury and remodeling, the myriad of agents that have been directly assessed as metabolic modulators have not been

found to have significant clinical benefit in the management of heart disease. The use of these agents has recently been reviewed,[16] and only some of the studies are discussed here to illustrate the overall lack of efficacy, inadequate sample size, and potential adverse effects associated with the administration of these metabolic modulators. Etomoxir is an irreversible inhibitor of mitochondrial carnitine palmitoyltransferase-1 (CPT-1) and thus results in a reduction in long-chain FAO. An initial pilot study suggested that etomoxir might improve myocardial function in patients with heart failure[38]; however, a subsequent controlled study was stopped prematurely because the drug was associated with hepatic transaminitis.[39] Another inhibitor of CPT-1 and CPT-2 is perhexiline, and one study showed improvement in maximum oxygen consumption and ejection fraction[40]; however, larger studies are needed before a conclusion can be made regarding the use of this drug in heart disease. Trimetazidine is a partial inhibitor of the β-oxidation enzyme 3-ketoacyl CoA thiolase and has been shown in small studies to improve symptoms and cardiac function in patients with heart failure.[41] Ranolazine has a similar structure to trimetazidine and is currently approved by the US Food and Drug Administration for treatment of stable angina. Although it can affect free fatty acid metabolism, its main mechanism of action might be related to inhibition of the late inward Na^+ channel. In the MERLIN-TIMI 36 trial (Metabolic Efficiency With Ranolazine for Less Ischemia in Non–ST-Elevation Acute Coronary Syndromes–Thrombolysis in Myocardial Infarction 36), ranolazine did not reduce hospitalization rates for heart failure among patients with acute coronary syndrome.[42] Finally, dichloroacetate increases myocardial glucose utilization by inhibiting pyruvate dehydrogenase kinase, which leads to increased activity of the mitochondrial pyruvate dehydrogenase. In a small study of patients with heart disease, dichloroacetate increased stroke volume and myocardial efficiency[43]; however, more substantial studies are needed to characterize the role of dichloroacetate in patients with heart disease.

Creatine Kinase and High-Energy Transfer

The creatine kinase (CK) reaction is the prime energy reserve that provides a rapid source of ATP and facilitates its delivery from the mitochondrial site of production to sites of use, including the myofibrils in the heart.[44] Although the heart is a high-energy–consuming organ, human genetic mutations linked to creatine deficiency usually result in neurological deficits.[45] In parallel, the genetic disruption of whole-body creatine synthesis in the mouse had no detrimental effects on exercise capacity, cardiac workload, or adaptation to ischemia-reperfusion injury,[46] and overexpression of the creatine transporter, or exogenous creatine supplementation in mice, showed no cardiac benefit.[47] Nevertheless, in a large-animal model, reduced CK ATP delivery was associated with impaired myocardial contractile function,[48] and in human heart failure, morbidity and mortality are linked to impaired CK metabolism and flux.[49,50]

Regulation of Function and Metabolism: The Role of PTMs

It has been increasingly clear that there is cross talk and signaling between the mitochondria, the cytosol, and the nucleus. Posttranslational modifications are a primary mechanism by which the mitochondria communicate with the rest of the cell.

Acetylation

Nutrient overload is linked to mitochondrial dysfunction and to the cardiovascular risk factors of obesity and diabetes mellitus.[27,51] Conceptually, perturbations in mitochondrial metabolic intermediates, such as acetyl-CoA, which itself can function as a direct posttranslational substrate to modify mitochondrial proteins through acetylation, could link these pathophysiological effects.[52,53] Additional short-chain carbon metabolic intermediates, including succinyl groups and malonyl groups, can also bind to and modify protein lysine residues.[45,54] Our knowledge of the regulation of these latter modifications is too preliminary to expand on,[45,54,55] and this section will focus on the role of acetylation, as a nutrient-dependent mechanism, in the regulation of mitochondrial function.

Enzymatic and Nonenzymatic Control of Protein Acetylation

There are 3 major acetyltransferase families, and member proteins from each group have been implicated in the control of cellular homeostasis.[56] Deacetylase proteins are similarly grouped into distinct classes.[57] Class III deacetylases are NAD^+ dependent and function as sensors of the energetic status of the cell in response to the subcellular compartment levels of NAD^+ and nicotinamide or to the ratio of NAD^+ to NADH.[35,58,59] Recent findings have highlighted novel mechanisms that regulate levels of NAD.[60–62] The class III enzymes are termed sirtuins, and 7 family members (Sirt1 through Sirt7) are evident in mammalia.[63] Sirt1, 2, and 3 have the most robust deacetylase activity and predominantly function in the nuclear (Sirt1), cytoplasmic (Sirt1 and 2), and mitochondrial (Sirt3) compartments, respectively. Because the focus of this section is on mitochondrial PTMs, it will focus on Sirt3.

The counterregulatory acetyltransferase enzyme system is less well characterized, although nuclear Gcn5 and p300 counter the actions of Sirt1.[64] The process of protein acetylation in the mitochondria is even less well understood, although GCN5L1 has been identified as a critical molecular component of this program, and its functional role is beginning to be explored.[53,65] Recently, the mitochondrial protein acetyltransferase, acetyl-CoA acetyltransferase 1 (ACAT1), which functions in ketogenesis to combine 2 acetyl-CoA molecules,[66] has been found to regulate the pyruvate dehydrogenase complex as a canonical acetyltransferase.[67] This finding could open the door to expanding our understanding of acetyltransferase functioning within mitochondria.

Concurrently, the recognition of nonenzymatic acetylation of proteins in the presence of acetyl-CoA is evident,[68] and denatured mitochondrial proteins undergo acetylation in the presence of acetyl-CoA.[65] Furthermore, elevated levels of acetyl-CoA, coupled to the alkaline mitochondrial pH, have been shown to promote nonenzymatic protein acetylation.[69] This concept of nonenzymatic protein acetylation might be operational in diabetes mellitus, in which metabolic inflexibility, which is defined as the inability to switch from fatty acid to glucose oxidation during the transition from the fasted to the fed state, results in part from the allosteric inhibition of pyruvate dehydrogenase by increased mitochondrial acetyl-CoA

levels.[70,71] The role of nonenzymatic protein acetylation has not been investigated extensively, although its potentially important regulatory role has been reviewed.[72] Interestingly, analysis of the mitochondrial acetylome, under various nutrient conditions and in the presence or absence of Sirt3, shows evidence compatible with nonenzymatic and enzymatic control of the mitochondrial acetylome.[73]

Mitochondrial Sirt3 and the Heart

Although Sirt3 functions predominantly in mitochondria,[74] data do support extramitochondrial deacetylase activity.[75–77] The depletion of Sirt3 has a subtle phenotype[78] that is unmasked in response to prolonged fasting,[79] after chronic perturbations in caloric intake,[80–82] and in response to redox stress.[83] Numerous proteomic approaches have been used to identify substrates of Sirt3 deacetylation, and the vast majority of proteins with alternations in acetylation are found within mitochondria.[73,84,85] The functional characterization of these proteins shows that Sirt3-mediated deacetylation regulates numerous aspects of mitochondrial function, including the regulation of enzymes that control β-oxidation, branch-chain amino acid metabolism, ketone biology, the ETC, ATP production, the urea cycle,[73,79,85,86] and ROS catabolism.[74,87]

In light of the high energy demand of the heart and based on the Sirt3 targets characterized to date, the disruption of Sirt3 would be expected to have cardiac consequences. Despite this, young Sirt3 knockout mice do not have any obvious phenotype[88] and furthermore display normal exercise performance.[89] However, consistent with a "fine-tuning" function, aging Sirt3 knockout mice develop cardiac dilatation,[88] and pressure overload results in maladaptive cardiac hypertrophy.[88,90] The mechanisms underpinning these pathologies align with established functions attributable to Sirt3, including increased generation of ROS.[88,90] Conversely, Sirt3 overexpression promotes antiapoptotic programs in cardiomyocytes,[76] and cardiac-restricted Sirt3 transgenic mice exhibit enhanced ROS scavenging.[90] An interesting additional mechanism whereby Sirt3 deficiency could potentially contribute to the pathophysiology of cardiac hypertrophy is its regulatory role in controlling fatty acid metabolism.[79] Because the loss of metabolic plasticity with the downregulation of FAO is synonymous with cardiac pressure overload–mediated decompensation,[12,17] it is conceptually possible that the downregulation of FAO in Sirt3 knockout mice could play a role in the pressure-overload and aging maladaptive phenotype in the heart. However, this needs to be delineated further, because high-fat feeding has been shown to increase cardiac FAO in parallel with downregulation of Sirt3.[91]

Because regulatory control of mitochondrial protein acetylation is nutrient-level and redox-potential dependent, it is conceivable that primary perturbations within mitochondria that can modulate metabolic intermediates or redox potential could initiate changes in the acetylome. This concept has been explored in the heart in response to genetic perturbations associated with cardiovascular pathology in which disruption of frataxin, cyclophilin D, and components of the ETC result in either basal or excessive pressure-overload–induced cardiac dysfunction and are associated with reduced NAD^+-NADH ratio and increased mitochondrial protein acetylation.[13,92,93] In primary cardiomyocytes, frataxin and complex I disruption of the acetylome are corrected in parallel with improvement in mitochondrial function after Sirt3 induction.[13,92] Although incompletely characterized, these data support the concept that control of acetylation by intrinsic mitochondrial functioning could, via a feedback loop, affect global mitochondrial functioning via mitochondrial acetylome regulation.

Future Directions in Understanding the Mitochondrial Acetylome

Advances in proteomics have enhanced our understanding of both the static and dynamic alteration of the mitochondrial acetylome.[73,94] Additionally, these studies have identified site-specific changes in lysine residue acetylation that modulate protein function, stability, localization, and allosteric interactions or control synergistic PTMs.[79,84,95,96] Moreover, the stoichiometry of proteins and the domains that surround substrate protein lysine residues could play important regulatory roles in the interaction of acetylase and deacetylase enzymes,[73] and the further characterization of the acetylome-modifying enzymes themselves might expand our understanding of the role of acetylation in controlling mitochondrial function.[53,74,94]

An area of some functional discrepancy has also arisen with respect to the acetylation of specific targets within a pathway compared with the global functioning of the canonical pathway in response to acetylation. This is most vividly illustrated where FAO is increased in the presence of excess fat and mitochondrial protein acetylation,[52,97] in contrast to studies that have shown direct deacetylation of lysine residues on FAO enzymes resulting in activation of enzyme activity.[79,98] The mechanisms underpinning these effects and whether this might be a result of tissue-distinct regulatory cues need to be explored.

Finally, although the role of acetylation in modifying individual proteins is the main focus of this section, data are emerging to show that the overall function of mitochondrial quality control and integrity, which are also modulated by nutrient levels and redox stress, including mitochondrial turnover (mitochondrial dynamics, mitophagy, and biogenesis)[53,64,99,100] and redox- and proteotoxic-stress amelioration effects,[101,102] might be regulated by the mitochondrial acetylome.[53] The complexity of this regulation is further underscored where cross talk between different PTMs functions in concert to regulate protein function, as has been shown by concomitant modifications in acetylation and phosphorylation.[103]

Phosphorylation

As recently reviewed, there is extensive phosphorylation of proteins in the mitochondrial matrix, as well as in the mitochondrial electron transport complexes.[104,105] A number of recent studies have reported that there are several hundred phosphorylated proteins in cardiac mitochondria.[106,107] There are also sex differences in phosphorylation of mitochondrial proteins.[108] Many of the phosphorylated mitochondria proteins are outer mitochondrial proteins, which are likely phosphorylated by cytosolic kinases and have been shown to regulate mitochondrial dynamics and cell death pathways. As discussed previously,[104] the occupancy or fraction of the protein that is phosphorylated might be low for many of these proteins, and it is possible that these many of these low-level

modifications are of little or no functional consequence. It is also unclear to what extent phosphorylation of mitochondrial matrix proteins occurs in the matrix as opposed to before import into the matrix. Furthermore, with the exception of the PDH (pyruvate dehydrogenase) and BCKDH (branched-chain α-keto acid dehydrogenase) kinase and phosphatase, little is known about the kinases and phosphatases responsible for mitochondrial phosphorylation. O'Rourke et al[105] recently reviewed the evidence for mitochondrial localization of other kinases. Furthermore, although a large number of phosphorylated mitochondrial proteins have been identified, very few phosphorylation sites have been demonstrated to alter enzyme or protein activity. It has been proposed that cAMP generated in the mitochondria activates mitochondrial protein kinase A to regulate ATP production.[109] However, recent studies have found that alterations in mitochondrial cAMP and protein kinase A do not contribute significantly to acute calcium stimulation of oxidative phosphorylation.[110]

Given that extensive phosphorylation has been identified in the mitochondria, it is tempting to speculate that changes in mitochondrial phosphorylation regulate mitochondrial function. However, it will be important for future studies to better define the function consequences of these sites of phosphorylation and to define the kinases and phosphatases that regulate their phosphorylation.

S-Nitrosylation

S-nitrosylation (SNO) is the covalent attachment of nitric oxide (NO) moiety to a protein thiol group. As recently reviewed,[111–113] SNO is a redox-dependent modification that is suggested to alter cell function by altering protein or enzyme activity, altering protein localization, shielding critical cysteine residues from oxidation, altering protein stability, altering binding partners, and competing with other PTMs. An increase in oxidative stress leads to a decrease in protein SNO, which thereby alters the SNO/ROS balance. ROS leads to the consumption of NO, and thus, cardiac-specific overexpression of SOD leads to an increase in NO bioavailability.[114] Another mechanism by which an increase in oxidative stress reduces NO/SNO signaling is by uncoupling of NOS. Alterations in NOS signaling have been proposed to predispose one to cardiovascular disease.[115] Cardioprotection is associated with a modest increase in SNO, and the majority of the proteins that exhibit an increase in SNO are mitochondrial.[116] This might be related to the redox environment of the mitochondria. Changes in cell redox can alter the generation of NO, the lifetime or bioavailability of NO, and the reactions that lead to protein SNO and denitrosylation. A key cysteine in the mitochondrial ATP synthase was shown to undergo multiple redox modification, and the extent of different modifications differed in dyssynchronous heart failure compared with cardiac resynchronization therapy.[117]

Calcium Transport

The electrochemical gradient across the IMM is the driving force for calcium transported across the mitochondria inner membrane by the recently identified[118,119] mitochondrial calcium uniporter (MCU). Uptake into the mitochondria of small physiological levels of calcium is thought to regulate mitochondrial metabolism and ATP production.[120–123] In the heart, an increase in contractility is mediated by an increase in the cytosolic calcium transient. The increase in cytosolic calcium is transmitted to the mitochondria via Ca uptake into mitochondria, which leads to activation of the calcium-sensitive mitochondrial dehydrogenases[124] and several complexes of electron transport, thereby increasing ATP production as needed for the increase in work.[120] Under pathological conditions of high cytosolic calcium (calcium overload), mitochondria are capable of taking up large amounts of calcium, which leads to the opening of the mitochondrial permeability transition pore (mPTP), a large conductance channel in the IMM[125,126] (Mitochondria and Cell Death). The sustained opening of this transition pore is a trigger for cell death.[126]

As reviewed recently, the MCU exists in a multiprotein complex with several proteins that regulate its activity.[127–132] Calcium efflux from cardiac mitochondria occurs via the Na-Ca exchanger (NCXL) (see Boyman et al[133] for a recent review). Calcium transits the outer mitochondrial membrane (OMM) via the voltage-dependent anion channel. Mitochondrial Na-Ca exchange has been shown to regulate mitochondrial calcium levels and to connect mitochondrial calcium to intracellular sodium, such that the rise in sodium that occurs during hypertrophy and heart failure is reported to lead to alterations in mitochondrial calcium that lead to altered redox and metabolism.[134,135] There are recent data suggesting that alterations in mitochondrial calcium can contribute to the development of arrhythmias.[134,136]

Recently, several groups developed MCU-knockout mice or mice without a functional MCU to study the role of mitochondrial calcium in modulating metabolism and cell death.[137–141] Because it is generally assumed that mPTP opening and subsequent cell death is initiated by calcium influx into the mitochondria via the MCU, it was hypothesized that the MCU-knockout hearts would have a reduced mPTP opening and reduced cell death after ischemia. There was consistency among the different groups in that mitochondria from the MCU-knockout hearts did not take up calcium and did not undergo calcium-activated mPTP[137,139,140]; however, there were interesting differences regarding whether these mice were protected from ischemia-and-reperfusion–mediated death. In the mice in which MCU was knocked out or mutated before birth, the hearts did not show a decrease in infarct size after ischemia-reperfusion.[137,141] In contrast, the mice in which loss of MCU was induced in adults by administration of tamoxifen showed smaller infarcts after ischemia and reperfusion.[139,140] One possible explanation for these differences is that when MCU is deleted before birth, compensatory mechanisms develop that somehow modify cell death pathways such that loss of MCU is not protective. A role for compensatory mechanisms is also consistent with the observation that loss of MCU is lethal on a C57B6 background.

Mitochondria and Cell Death

Before the 1980s, cell death was viewed as a passive process. At odds with this concept were long-standing observations that specific cells die at specific times during development in multicellular organisms ranging from worms to mammals.[142] However, it was not until the identification of a small

network of genes that modulate developmental cell death in *Caenorhabditis elegans* that the concept of regulated cell death came into focus.[143] By the 1990s, the descendants of these genes were recognized to also mediate apoptotic cell death in adult organisms, including humans.[144] By the turn of the century, it became clear that a large proportion of necrotic cell deaths, thought to be the last bastion of passive cell death, were actually highly regulated.[145–148] In addition to apoptosis and necrosis, other regulated death programs (defined by morphology or the context in which they occur) likely exist,[149] including a form of cell death associated with autophagy (autosis).[150] What regulated forms of cell death share in common is a process mediated by signaling pathways whose components are constitutively present in the cell. These hardwired pathways remain inactive, however, until receipt of a "death signal" that originates from outside or inside the cell.

Apoptosis and necrosis have been studied most intensively. Although they share inciting death stimuli and are mediated by overlapping pathways, they differ in morphology and consequences to surrounding tissue.[151] Specifically, apoptosis is a stealth form of cell death, because plasma membrane integrity is maintained until the fragmented cellular corpses are eliminated by phagocytosis. In contrast, plasma and organelle membrane breakdown is a defining feature of necrosis and can be actively mediated. The end result in necrosis is the release of inflammatory mediators that cause collateral tissue damage in a paracrine manner and through the recruitment of leukocytes. On the basis of traditional pathological analysis, the major form of cardiomyocyte death in the infarct zone is thought to be necrosis,[152] while a delayed wave of apoptosis takes place in the peri-infarct region, especially with reperfusion.[153,154] Genetic experiments in mice have established that regulated necrosis and apoptosis both play important roles in the generation of the infarct (examples include those in references 95,147, and 155–163). In dilated cardiomyopathy, low but clearly elevated levels of cardiomyocyte apoptosis take place and are an important component in the pathogenesis of this syndrome.[164] Necrosis has also been reported to contribute to heart failure but has been less well studied.[148]

Apoptosis and necrosis can each be induced through 2 general pathways, one involving cell surface "death" receptors and the other the mitochondria.[144,151,165] Even when the signals are initiated through death receptors, the mitochondria are often part of a critical amplification loop. Regardless of the initiating pathway, the end game in apoptosis is to activate caspases, a class of cystinyl proteases that cut after aspartic acid residues. Caspases then proteolyze multiple cellular substrates to bring about the demise of the cell. The molecular goal in necrosis, on the other hand, depends on the initiating pathway. Induction of necrosis through the death receptor pathway (necroptosis) is mediated through activation of receptor interacting protein (RIP) 1 and RIP3, homologous serine/threonine kinases whose targets are an area of active investigation.

Mitochondria have been recognized as playing a central role in both apoptotic and necrotic cell death. The triggering event in mitochondria-mediated apoptosis is permeabilization of the OMM, which allows the release of apoptogens, including cytochrome c, SMAC/DIABLO, Omi/HtrA2, AIF, and EndoG.[165] What these proteins share in common is that they perform healthy functions within the mitochondria but are toxic in the cytosolic compartment. For example, in healthy cells, cytochrome c participates in electron transport at the IMM as part of oxidative phosphorylation. In contrast, once cytosolic during apoptosis, cytochrome c binds Apaf-1 to trigger assembly of the apoptosome in which procaspase-9 is activated. OMM permeabilization during apoptosis is promoted by BAX and BAK, pro-cell death members of the BCL-2 family of proteins.[166] Although it is not known precisely how these proteins bring about permeabilization (eg, one model involves pore formation), it is clear that homo-oligomerization and hetero-oligomerization are important. BAX and BAK are regulated primarily through changes in their conformations. In the case of BAX, which resides in the cytoplasm of healthy cells in an inactive conformation, conformational activation[167,168] is brought about by direct binding of BIM or a truncated form of BID (tBid), which are members of the BH3-only arm of the BCL-2 family. The function of BH3-only proteins is to bring death signals to BAX and BAK from other pathways in the cell. Activation of BAX exposes a transmembrane domain in its 9th α-helix that has a predilection for the OMM and presumably facilitates BAX mitochondrial translocation. BAK resides constitutively in the OMM and is thought to be activated in a similar fashion, although this has been studied in less depth. Antiapoptotic BCL-2 proteins such as BCL-2, BCL-xL, and MCL-1 inhibit BAX and BAK by functioning as sinks for BIM and tBid and possibly also through direct interactions with BAX and BAK.

The triggering event in mitochondria-mediated necrosis is the sustained opening of mPTP in the IMM.[151] In healthy cells, the OMM is impermeant to apoptogens but allows the passage of ions and small molecules. Opening of the mPTP during necrosis results in rapid dissipation of the proton gradient across the IMM that is generated by pumping of protons into the intermembrane space during oxidation of substrates in the Krebs cycle. Because this transmembrane proton gradient is needed to drive ATP synthesis, mPTP opening abruptly stops production of new ATP. To further compound this energetic deficit, ATP consumption continues largely unabated during necrosis.[169] In contrast, apoptotic cells shut down ATP-requiring functions such as DNA repair, translation, and proteasome function[170–172] and experience less reduction in ATP synthesis. A second consequence of mPTP opening during necrosis is the ingress of water down its osmotic gradient into the solute-rich mitochondrial matrix. This causes matrix swelling, which results in expansion of the redundant IMM and sometimes rupture of the OMM, which lacks redundancy. Rupture of the OMM sets up the possibility that apoptogens could gain access to the cytoplasm in necrosis (albeit via OMM rupture rather than permeabilization) and trigger caspase activation.[147] Given the cataclysmic events that result from cessation of ATP synthesis, the extent to which subsequent engagement of the downstream apoptosis signaling contributes to cell death in necrosis is unclear.

The composition of the mPTP has been an area of great controversy.[173] The pore has often been depicted as a complex that involves the voltage-dependent anion channel in the OMM and the adenine nucleotide translocase (ANT) in

the IMM. Genetic studies, however, have demonstrated that neither the voltage-dependent anion channel[147] nor ANT[174] is required for pore opening. Similarly, the mitochondrial phosphate carrier in the IMM, more recently hypothesized to be part of the mPTP, has proved to be dispensable.[175,176] What then is the mPTP? Recent work suggests the unanticipated result that a core component is the F_1-F_0 ATP synthase itself.[177–179] Although these data are exciting, additional studies will be required for in vivo proof.

The best characterized stimulus for mPTP opening is an increase in the concentration of Ca^{2+} in the mitochondrial matrix.[180] The effects of increased [Ca^{2+}] on mPTP opening are sensitized by oxidative stress, increases in phosphate, and decreases in ATP and ADP.[180–182] These conditions operate during ischemia and reperfusion.[183] The binding site through which Ca^{2+} triggers mPTP opening is not known, however. A critical facilitator of mPTP opening is cyclophilin D, a peptidyl prolyl isomerase in the mitochondrial matrix.[160,184] Although it is known that cyclophilin D binds the F_1-F_0 ATP synthase,[178] and it has been reported that cyclophilin D prolyl isomerase activity is required for facilitation of mPTP opening,[184] the precise mechanism is not understood. The drug cyclosporin A, which binds cyclophilin D, inhibits mPTP opening and necrosis.[185] Although not an essential component of the mPTP, ANT also functions as a positive regulator of pore opening.[174] Recently, the proapoptotic proteins BAX and BAK were found to be critical mediators of primary necrosis.[163,186] Mice lacking BAX and BAK or BAX alone exhibit markedly decreased cardiac necrosis, apoptosis, and infarct size after ischemia-reperfusion in vivo.[158,163] Analysis of BAX mutants shows that its apoptotic and necrotic functions are distinct. Current evidence supports 2 nonmutually exclusive models in which BAX functions as an OMM component of the mPTP or facilitates necrosis indirectly by promoting mitochondrial fusion.

Many questions remain concerning the mitochondrial events that mediate cell death. First, the complete composition of mPTP is not clear at this point. Second, the upstream signaling that feeds into both necrotic and apoptotic programs at the mitochondria remains incompletely understood, especially in the case of ischemia-reperfusion. Third, the molecular connections linking apoptotic and necrotic programs at the mitochondria and the factors that determine how a specific cell will die are not known in any depth.

Despite these deficits in knowledge, inhibition of cell death has been contemplated, especially for ischemic syndromes. We will limit the discussion here to 2 points. First, a small clinical trial of cyclosporin A, administered at the time of percutaneous coronary intervention for ST-segment–elevation myocardial infarction, suggested reductions in infarct size.[187] A follow-up study in a larger number of patients, however, failed to show smaller infarcts or improvement in clinical outcomes.[187a,187b] Given genetic data implicating mPTP opening as important in necrotic cell death during myocardial infarction,[160,184] possible interpretations include that cyclosporin A is not an optimal small molecule in this situation or that additional necrosis pathways require inhibition.[184a] Second, given that necrosis and apoptosis both contribute to the pathogenesis of myocardial infarction, selection of a therapeutic target such as BAX, which mediates both forms of cell death,[163,186] is worthy of consideration.

Generation of ROS

During electron transport, if there is any leakage of electrons, it can lead to the generation of ROS, and mitochondria are one of the major cellular sources of ROS. Mitochondria also contain antioxidant mechanisms to remove ROS. At low levels, ROS can act as a signaling molecule, whereas higher levels can lead to irreversible damage to mitochondria and cells and are a major contributor to cardiovascular disease.

In mitochondria, ROS formation results from sporadic, possibly undesired reactions that occur, especially at the level of the ETC.[189–191] Besides these occasional processes, mitochondria also contain enzymes that catalyze H_2O_2 generation as the obligatory product.[192]

The ETC drives electrons from reduced coenzymes [NADH(H^+) and $FADH_2$] to oxygen that undergoes the complete reduction to water in the terminal reaction catalyzed by complex IV (ie, cytochrome c oxidase). A minor fraction (≈0.1%) of the electrons flowing through the ETC are suggested to cause the partial reduction of O_2 into superoxide.[189] In particular, flavins or quinones of the first 3 complexes are able to act as single-electron donors resulting in superoxide formation, especially under conditions that decrease the flow of electrons toward complex IV, where O_2 is fully reduced to H_2O.[189,190] Notably, ROS formation can also result from reverse electron flow. Recently, this concept has been supported by demonstrating in vivo that succinate accumulated during ischemia is oxidized during reperfusion, resulting in large ROS formation that is likely attributable to the reverse electron flow within complex I.[193]

ROS formation is favored by high mitochondrial membrane potential (ie, low ATP synthesis), large NADH(H^+), or when electron flow is hampered by alterations in respiratory complexes. Conversely, a decrease in ROS levels should follow the acceleration in electron flow caused by mitochondrial uncoupling,[194] yet conditions have been reported in which mitochondrial uncoupling and $\Delta\psi_m$ dissipation are associated with increased ROS formation.[135,195] According to the model of redox-optimized ROS balance,[196] this apparent paradox might be explained by a concomitant depletion of the antioxidative capacity, which would result in H_2O_2 accumulation despite decreased formation of superoxide by the ETC.[197]

Increased ROS formation is also associated with the uncoupling-like effect generated by opening of the mPTP. Indeed, this process has been proposed to amplify an initial oxidative stress through the so-called ROS-induced ROS release.[191] ROS can trigger PTP opening through oxidative modifications of mitochondrial proteins involved in PTP formation and control, such as FoF1 ATP synthase e[198] or cyclophilin D.[199] However, despite evidence that ROS formation follows PTP opening,[200,201] the underlying mechanisms have not yet been elucidated. On the other hand, a slight increase in ROS formation resulting from opening of mitochondrial K^+ ATP channels has been proposed to prevent mPTP opening and elicit cardioprotection.[202–204] A similar process could contribute to protection induced by preconditioning or postconditioning that is abrogated by antioxidant treatment.[188,205,206] Therefore, the

notion that mild ROS accumulation increases the resistance to oxidative stress[207] might be explained by opposite effects on the susceptibility to PTP opening elicited by slight and large ROS formation, respectively.

Superoxide that does not cross the IMM is rapidly dismutated into the freely permeable H_2O_2 by Mn-superoxide dismutase (Mn-SOD). The finding that Mn-SOD–deficient mice develop ROS toxicity and dilated cardiomyopathy[208,209] underscores the importance of ROS in this pathology and mitochondria as their source and target. This concept is further supported by the beneficial effects afforded by targeting catalase expression in mitochondria.[210–212]

Besides respiratory chain complexes, several other mitochondrial enzymes have been described as potential ROS producers. These include the flavin containing glycerol-3-phosphate-dehydrogenase, proline-dehydrogenase and dihydroorotate-dehydrogenase at the outer leaflet of the IMM; the electron transfer flavoprotein-ubiquinone (ETF:Q) oxidoreductase system of FAO within the IMM; and pyruvate- and 2-oxoglutarate dehydrogenase within the mitochondrial matrix.[213] However, the contribution of these enzymes to the overall ROS production of mitochondria within a given cell is difficult to establish. In fact, as is also the case with respiratory complexes, loss-of-function approaches (ie, pharmacological inhibition or genetic deletion) would inevitably hamper the physiological functions of these vital proteins, jeopardizing energy metabolism, ionic homeostasis, and cell viability. Convincing demonstration that mitochondria generate ROS in vivo is also provided by interventions targeting mitochondrial enzymes such as p66Shc and monoamine oxidases (MAOs) that generate H_2O_2 as a direct and obligatory product.

In response to various stress stimuli, the cytosolic adaptor protein p66Shc translocates to mitochondria, where it catalyzes H_2O_2 formation by means of electron transfer from cytochrome c to oxygen.[214] Indeed, ROS generation is reduced in cells lacking p66Shc and in p66Shc$^{-/-}$ mice, whose lifespan is increased by 30%.[215,216] Furthermore, genetic deletion of p66Shc protects against ischemia-reperfusion injury and diabetes mellitus–induced cardiovascular derangements.[192,217]

The 2 isoforms of MAO, A and B, are flavoenzymes located in the OMM. MAOs catalyze the oxidative deamination of catecholamines, serotonin, and biogenic amines generating the corresponding aldehydes, H_2O_2 and ammonia. H_2O_2 and aldehydes[206] produced by MAO have been shown to synergize in disrupting mitochondrial function associated with loss of function and viability of the heart.[192] In addition, ammonia might stimulate ROS formation by dihydrolipoyl dehydrogenase, the E3 component of pyruvate and oxoglutarate dehydrogenase.[218] Interestingly, in human atrial biopsy samples, MAO has been shown to produce 10 times more H_2O_2 than the respiratory chain, and its expression is correlated with an increased risk for postoperative atrial fibrillation.[219] Major advantages of investigating the role of MAO in oxidative stress are given by a defined molecular structure, specific substrates, and clinically available inhibitors. However, the substrates used and the mechanisms of activation under injury conditions are still not clear. In addition, the clinical use of MAO inhibitors in cardiovascular diseases is perceived as problematic because of a hypertensive reaction that occurs when selective MAO-A inhibition is combined with intake of tyramine-rich food, such as aged cheese and alcoholic beverages. Conversely, MAO-B inhibition is devoid of this potential risk.[220]

The list of dedicated enzymes for ROS formation in mitochondria includes nicotinamide adenine dinucleotide phosphate oxidase 4 (NOX4).[221] At variance from other NOX isoforms localized to the plasma membrane, NOX4 displays a preferential localization to intracellular sites, appears to be constitutively active, and generates H_2O_2 in preference to superoxide.[222,223] Because localization to cardiac mitochondria has been established on the basis of reactivity to antibodies that were not tested in NOX4$^{-/-}$ cardiomyocytes, further studies are necessary. In addition, the role in the cardiovascular system is debated, because NOX4 appears to cause both beneficial and detrimental effects in models of cardiac pressure overload.[28,29,77,221]

As in the rest of the cell, in mitochondria, ROS generation is counterbalanced by efficacious removal systems. Besides superoxide dismutation by Mn-SOD, peroxide handling is performed by a thiol redox system centered on glutathione (GSH and GSSG in its reduced and oxidized form, respectively) and thioredoxin (Trx).[224–226] H_2O_2 is reduced to water by glutathione peroxidases (Gpx1 and 4) and peroxiredoxin 3 (Prx3), which is maintained in the active reduced form by Trx. In addition, GSH is used by the mitochondrial glutathione-*S*-transferase K to detoxify products of oxidative damage, such as α,β-unsaturated aldehydes and alkyl hydroperoxides, and by glutaredoxin 2, which catalyzes the formation and reversal of protein-GSH mixed disulfides.[225]

The oxidized forms of glutathione and Trx resulting from Gpx and Prx catalysis are reduced by the corresponding reductases at the expense of NADP(H^+). The mitochondrial $NADP^+$ pool is reduced by malic enzyme, $\Delta\psi_m$-dependent nicotinamide nucleotide transhydrogenase, and Ca^{2+}-modulated isocitrate dehydrogenase. Therefore, the $NADP^+$/NADP(H^+) ratio links oxidative metabolism and mitochondrial function with ROS signaling and antioxidant activities.[197] Prx3 is responsible for >90% of H_2O_2 removal in mitochondria.[227] However, because Prx3 is highly susceptible to oxidation, under conditions of severe oxidative stress such as myocardial ischemia and reperfusion,[228] Gpx1 might become the major sink for H_2O_2.[225] Prx3 overexpression confers increased resistance to ischemia-reperfusion injury,[229] which lends further support to the relevance of mitochondria-generated ROS in cardiac diseases.

The occurrence and the relevance of ROS formation in mitochondria are supported by direct methods for in vivo detection,[230] effects of targeted antioxidants and enzymes,[231] and decreased ROS accumulation after inhibition or deletion of mitochondrial ROS sources.[232] The majority of studies relate mitochondrial ROS formation with cell injury, which suggests that beneficial effects are afforded by preventing ROS accumulation in a wide array of cardiovascular diseases, such as ischemia-reperfusion injury, heart failure, aging, and diabetic cardiomyopathy.[190,192,217,233,234]

Although it is undeniable that high levels of ROS impair the function and viability of any cell type, a large body of evidence indicates that ROS generated within mitochondria are involved in signaling processes that are crucial for optimal

response to physiological and pathological stimuli.[192,235–237] Indeed, several reports document the crucial role of mitochondrial ROS generation in a wide variety of cardiomyocyte functions. The physiological role of mitochondrial ROS is likely to be linked to PTMs of proteins, especially at the level of cysteine residues.[238] For instance, SNO has been recognized as a cardioprotective mechanism that prevents irreversible oxidation of proteins.[225,230,239] In addition, signaling pathways involving protein phosphorylation are modulated by oxidation of critical cysteines, especially in protein phosphatases.[240] In addition to these short-term responses, mitochondrial ROS are involved in long-lasting changes by acting on transcriptional factors, such as hypoxia-inducible factors and nuclear factor erythroid 2–related factor 2 (Nrf2).[226,241–243]

Far from always being beneficial, a decrease in mitochondrial ROS levels could be detrimental. A large increase in glutathione content or the administration of *N*-acetylcysteine was shown to elicit mitochondrial oxidation and cytotoxicity despite a decrease in ROS levels.[244] Suppression of mitochondrial ROS generation by mitochondria-targeted catalase hampered autophagy, which worsened heart failure caused by deletion of mitofusin 2.[245] Interventions aimed at reducing mitochondrial ROS levels, such as expression of dominant negative Nox,[149] deletion of p66Shc,[246] or ablation of thioredoxin-interacting protein,[247] were found to exacerbate the mild injury induced by ischemia-reperfusion protocols of short duration. This paradoxical notion, which contrasts with protection by antioxidant treatments in prolonged episodes of ischemia-reperfusion, suggests that mitochondrial ROS are involved in triggering self-defense mechanisms. Supporting this concept, antioxidants abrogate the powerful protection of both ischemic preconditioning and postconditioning.[203,204,206] In this respect, the term *mitohormesis* has been introduced to describe the J-shaped curve whereby low doses of mitochondrial ROS trigger beneficial adaptive responses that are replaced by detrimental processes at high doses.[241,248] Although this concept appears to accommodate the protective effects of exercise and calorie restriction,[248] especially in clinical settings, methods are not available to define the threshold that separate beneficial from harmful ROS levels. Other relevant issues to address are the interactions among the various ROS sources and the conditions involved in local compartmentalized ROS formation compared with diffusion of ROS and oxidized products to the entire cell and surrounding tissues.

Mitochondrial Quality Control

Fission/Fusion/Mitochondrial Dynamics

As cells and organisms reproduce, their mitochondria divide to repopulate the progeny. Mitochondria also divide and fuse back together in nondividing, quiescent, and postmitotic cells such as cardiomyocytes and neurons; however, the rates in cardiomyocytes appear to be quite low. This continual fission and fusion cycle, a process also called mitochondrial dynamics, is known to be essential for the healthy maintenance of mitochondria and their host cells and organisms. Mitochondrial dynamics participate in mitophagy, apoptosis, differentiation, and a variety of stress responses. The adverse consequences that an interruption in the in vivo cardiomyocyte mitochondrial dynamics has on mitochondrial stress, mitochondrial biogenesis, and programmed cardiomyocyte death were recently demonstrated in side-by-side comparative studies after conditional genetic deletion of either cardiac mitochondrial fusion or fission pathways.[249]

Mitochondrial morphology reflects the relative rates of fission and fusion and can be visualized in fixed cells and tissues by immunostaining. Perturbing the ratio of fission and fusion rates will lead to either fragmented, punctiform mitochondria or excessively long or interconnected mitochondria. However, because mitochondrial morphology is dynamic, fission and fusion rates are best visualized in live cells. Quantification of mitochondrial fusion rates can be performed with photoactivatable green fluorescent protein (PAGFP) that is targeted to the mitochondrial matrix. When the mito-PAGFP is activated with a laser targeted to 1 mitochondrion, the PAGFP fluorescence is increased ≈100 times, and as this mitochondrion fuses with others, the fluorescence is diluted.[250] Quantification of this dilution rate reflects the organelle fusion rate independent of the fission rate and shows remarkable differences among cell types and changes, for example, early during apoptosis. An alternate approach to assess fusion uses 2 cell populations, one expressing green fluorescent protein in mitochondria and another expressing red fluorescent protein in the mitochondria. When cells from these 2 populations are fused with polyethylene glycol, the rate that the green fluorescent protein and red fluorescent protein merge to form yellow fluorescence reflects the mitochondrial fusion rate.[251] These techniques have been used extensively to characterize proteins that mediate the fusion process[252] and the physiological consequences of mitochondrial dynamics.[253]

The molecular machinery that mediates mitochondrial fusion uses large GTPases in the dynamin family.[252,254] Mitofusins (Mfn1, Mfn2) mediate fusion of the OMM, whereas Opa1 mediates fusion of the IMM. Mfn1, Mfn2, and Opa1 all require GTPase activity for fusion activity. Mfn1 and Mfn2 span the OMM, with most of the protein and the GTPase domain facing the cytosolic compartment. Opa1 is localized within the intermembrane space anchored to the IMM. Mitofusins and Opa1 usually work in concert to coordinately fuse both mitochondrial membranes. Opa1 activity is regulated by proteolytic processing, but how Mfn1 and Mfn2 are regulated is not yet clear.

Mitochondrial fission uses a large GTPase called Drp1 (dynamin-related protein 1), a homologue of dynamin that is well understood to mediate fission of endocytic vesicles from the plasma membrane. Like dynamin during endocytosis, Drp1 assembles into spirals that wrap around mitochondria and appear to constrict the inner and outer membranes during GTP cleavage to start the fission process. Drp1 exists free in the cytosol, from which it docks to mitochondrial fission sites by interacting with the OMM-spanning proteins Mff, Mid49, and Mid52.[252,254] Fis1 is a protein required for Dnm1 (a Drp1 orthologue)-mediated fission in yeast but is not required for fission in metazoans. Instead, Fis1 in metazoans participates in mitophagy.[255] Drp1 activity is regulated by phosphorylation at several sites on the protein. Phosphorylation at some sites stimulates fission, for example, during the cell cycle, and at other sites, phosphorylation inhibits fission. Interestingly,

endoplasmic reticulum tubules wrap around sites of mitochondria before their fission and might play a role in defining the site of mitochondrial fission or in assembling the fission complex at the proper location.[256]

Identification of these fission and fusion proteins has allowed generation of animal models and cell culture lines for the exploration of the physiological significance of mitochondrial dynamics. Mfn1, Mfn2, and Opa1 knockout mice are all embryonic lethal, which suggests that both fission and fusion are required for maintenance of mammalian embryos. However, fibroblasts generated from the embryos survive in culture, albeit with altered mitochondrial morphology and in some cases, metabolic deficits.[252] Cardiac myocyte–specific knockout of Mfn1 and 2 in adults causes cardiomyopathy. Interestingly, myocytes die after only 3 to 4 cycles of mitochondrial fission without opposing fusion.[83] Surprisingly, mitochondrial fusion is also linked to cardiac development. Through regulation of calcium levels and calcineurin, mitochondrial dynamics control Notch signaling and stem cell differentiation into cardiomyocytes.[257]

Mutations in several of the mitochondrial fusion genes have been identified as causing human disease.[252,254] Dominant optic atrophy, the most common form of hereditary blindness, is caused by haploinsufficiency in Opa1. Thus, retinal ganglion cells are highly dependent, and more so than other human tissues, on fusion of the IMM. Another example is mutations in Mfn2 that cause Charcot-Marie-Tooth disease type 2A. Understanding the intriguing tissue specificity of defects from mutations in mitochondrial dynamics genes, which have what might be considered housekeeping duties, remains a major challenge in discerning the roles of mitochondria in vivo. Because of the links of mitochondrial dynamics to human and animal health, there are efforts to drug the pathway. For example, mDIVI is a small molecule that inhibits Drp1 and mitochondrial fission that has been reported to have protective activity in numerous animal disease models, including heart ischemia-reperfusion injury.[258]

It is clear that mitochondria have to continually divide and fuse, but what are the essential roles of mitochondrial dynamics at the molecular level? Mitochondrial fission has been linked to damage avoidance through segregation of debris within mitochondria. As discussed elsewhere in this statement (Mitophagy and Mitochondrial Autophagy: Mechanisms, Overlaps, and Distinctions), mitophagy after fission allows the clearance of damaged mitochondria and selective elimination of damaged proteins.[253,259] Mitochondrial fission has been linked to apoptosis, which can also function as a severe form of stress response. Mitochondrial dynamics are also required for transport of mitochondria to proper locations within cells. On the other hand, fusion between mitochondria is thought to allow compensation to help rescue organelles from damage by the exchange of proteins and RNAs from 1 mitochondrion to another.[260] Mitochondria accumulate mtDNA deletions and mutations over time, and these mutations can generate mitochondrial proteins that are dysfunctional or misfolded. If mitochondria did not fuse, dysfunctional proteins could lead to serious loss-of-function consequences. However, fusion with another mitochondrion that might have mutations in other genes would allow compensation between organelles and avoid the serious consequences of mutation accumulation. Mitochondrial hyperfusion is a broadly active stress response that might facilitate such compensation.[261]

Mitophagy and Mitochondrial Autophagy: Mechanisms, Overlap, and Distinctions

Mitophagy, which literally means "eating mitochondria," is the term applied to the cellular mechanism for identifying and selectively eliminating dysfunctional mitochondria as part of the overall mitochondrial quality control process.[262] Mitophagy is essential to sequester and remove senescent or damaged mitochondria that could otherwise accumulate and become sources of cytotoxic ROS (Generation of ROS). Although the distal components of the mitophagy pathway (ie, autophagosomal engulfment of mitochondria and their transfer to lysosomes for degradation and component recycling) are shared with macroautophagy, the proximal events that detect and select dysfunctional organelles for targeted elimination are highly specific for mitophagy. Two central proteins driving this detection/selection process are the cytosolic E3 ubiquitin ligase Parkin[263] and the mitochondrial kinase PINK1,[56] encoded by genes (*PARK2* and *PARK7*, respectively) for which loss-of-function mutations have been linked to autosomal recessive forms of Parkinson disease.[264]

The discovery that PINK1 and Parkin interact to promote mitochondrial fitness[265,266] and the elucidation of mitochondrial stabilization of PINK1 as the initiating event in mitophagy[267,268] were central to our current understanding of the mechanisms underlying mitochondrial quality control. Briefly, healthy mitochondria maintain an electrochemical inner membrane gradient, $\Delta\psi_m$, that drives ATP production by the electron transport complex (Generation of ATP: Bioenergetics and Metabolism). Senescent mitochondria are unable to sustain a normal $\Delta\psi_m$, and damaged mitochondria can completely dissipate $\Delta\psi_m$, resulting in depolarization. Mitochondrial $\Delta\psi_m$ status is a key determinant of PINK1-Parkin pathway activity: Healthy, fully polarized mitochondria import and rapidly degrade PINK1, maintaining low kinase activity. On depolarization, however, PINK1 degradation is suppressed,[268] thereby increasing its abundance and promoting multiple PINK1 kinase–mediated events, including Parkin translocation to[269] and activation at[270,271] mitochondrial outer membranes. At the mitochondrion, Parkin ubiquitylates dozens of mitochondrial proteins,[272] thereby promoting autophagosomal engulfment of the damaged organelle. The overall result for the cell is selective mitophagic destruction of depolarized mitochondria.

Mitophagy is inextricably linked to mitochondrial dynamism (ie, mitochondrial fission and fusion). In many cells, mitochondria form highly interconnected reticular networks that are constantly remodeling through periodic fission and fusion. However, in adult cardiac myocytes, mitochondria fission/fusion is rare.[273] For this reason, it is likely that mitochondrial dynamism is dispensable for morphometric remodeling in hearts, but nevertheless, it plays an important role in cardiac mitophagic quality control through the process of Drp1-mediated asymmetric fission.[274] Accordingly, a mitochondrion in the early stages of senescence or one that has sustained moderate damage will segregate its dysfunctional components into 1 of the 2 daughter organelles generated

by a fission event. The damaged (and therefore depolarized) daughter mitochondrion will be promptly identified as such and removed via PINK1-Parkin mediated mitophagy, whereas the healthy daughter will rejoin the cellular mitochondria pool, likely by fusing with other similarly fit mitochondria. The particular role for Parkin-dependent versus Parkin-independent or "alternate" mitophagy mechanisms in healthy and diseased hearts is only beginning to be investigated.[275,276]

Mfn1 and Mfn2, so designated because they promote physical tethering between mitochondria and subsequent GTPase-dependent mitochondrial fusion, also have a role in mitophagy. In addition to promoting fusion of the healthy daughters after asymmetric fission, PINK1 kinase stabilization in damaged mitochondria results in phosphorylation of Mfn2 on 2 domains, thus conferring Parkin binding activity to this mitochondrial outer membrane protein and facilitating both Parkin translocation and its subsequent ubiquitination of mitochondrial proteins.[55] For this reason, hearts deficient in Mfn2 that do not exhibit defects in mitochondrial fusion (because Mfn1 is still present) instead develop a defect in mitochondrial quality control.[55,277]

There are surprising consequences of the mechanistic involvement of mitochondrial dynamism in mitochondrial quality control. For example, if mitophagy is malfunctioning but dynamism is intact, then the process of asymmetric fission will generate a highly dysfunctional daughter organelle that cannot be removed through the usual quality control process. Instead, the improperly retained damaged mitochondrion can fuse with (and by exchanging cellular components, thereby damage) normal mitochondria within the same cell. An example of fusion-mediated mitochondrial contagion was recently uncovered in Parkin-deficient *Drosophila* fruit fly heart tubes.[245] In this model, because fusion contributed to the spread of mitochondrial damage, heart failure was attenuated by cardiomyocyte-specific suppression of *Drosophila* mitofusin (MARF).

An important role for PINK1-Parkin–mediated mitophagy in normal functioning of the nervous system is clear from Parkinson disease.[278] Surprisingly, although genetic suppression of PINK1 or Parkin in fruit flies is detrimental to mitochondrial fitness and normal functioning of neural tissue, skeletal muscle, and myocardium,[279] germline gene ablation of the orthologous mouse genes evokes only modest phenotypes.[280] In mouse hearts, germline ablation of PINK1 and Parkin appears to produce only mild and slowly progressive basal cardiac dysfunction but increased sensitivity to ischemic injury.[84,93,281] Likewise, cardiomyocyte-specific Parkin ablation in adult mice provoked no detectable phenotype, and conditional cardiac Parkin overexpression had no detectable adverse consequences.[275] Thus, it is possible that PINK1-Parkin–mediated mitophagy is relatively unimportant to mitochondrial homeostasis in normal mammalian hearts. On the other hand, the absence of notable nervous system dysfunction (ie, Parkinson disease phenotypes) in these same mice,[280] evidence of compensatory upregulation of alternate E3 ligases in hearts of germline Parkin knockout mice,[245] and the cardiomyopathy that is evoked by cardiomyocyte-specific interruption of PINK1-Parkin signaling (through Mfn2 ablation)[55,277] suggest that this pathway of mitochondrial quality control could indeed be important under specific and as yet incompletely understood circumstances. The true role of PINK1 and Parkin in mammalian hearts might only be uncovered by the creation of new experimental models or by additional human genetic testing for rare PINK1 and Parkin mutations in clinical cardiac disease. Of note, Parkin can regulate fat uptake.[282]

If it is correct that maintaining mitochondrial quality is essential to cell health, then the absence of damaging mouse phenotypes in PINK1 and Parkin knockout mice and the focal degeneration of dopaminergic neurons (rather than larger systemwide effects) in Parkinson disease linked to human PINK1 or Parkin mutations suggest the presence of ≥1 alternative pathways of mitochondrial quality control.[283] Indeed, mitochondria can be eliminated independent of PINK1 and Parkin by an autophagic mechanism that uses the proapoptotic Bcl2 family proteins Nix and Bnip3 to target dysfunctional mitochondria and connect them to autophagosomes.[284] Conceptually, this mechanism resembles so-called mitoptosis, in which opening of the mPTP activates mitochondrial autophagy.[285] In this context, Nix and Bnip3 accumulate on damaged mitochondria, facilitate the permeability transition, and promote mitochondrial autophagy by serving as mitochondrial adaptor proteins that bind to autophagosomal LC3 or GABARAP.[286–289] Although Nix and Bnip3 are widely recognized for their proapoptotic effects in cardiac failure after pressure-overload hypertrophy and myocardial infarction, respectively,[290–293] the possibility that they also promote homeostatic mitochondrial autophagy in hearts merits further investigation.

Dissipation of $\Delta\psi_m$, aka mitochondrial depolarization, contributes to the signal for PINK1 stabilization and initiation of Parkin-mediated mitophagy. Evidence is accumulating that ROS, which are also markers of mitochondrial dysfunction, can play a similar role in Parkin-independent mitochondrial autophagy.[277,294] In vivo disruption of cardiomyocyte Parkin signaling by ablation of its Mfn2 mitochondrial receptor evokes a cardiomyopathy. As expected, normalization of ROS with mitochondria-directed catalase improves this mitophagic cardiomyopathy. In contrast, supersuppression of ROS (with mitochondria-directed catalase expressed at higher levels) is detrimental, both accelerating and exacerbating the cardiomyopathy.[277] These findings reveal an essential signaling function for mitochondria-derived ROS in compensatory mitochondrial autophagy pathways induced when the Parkin pathway is interrupted.

Protein Turnover Independent of Mitophagy

As discussed in the section on Mitophagy and Mitochondrial Autophagy: Mechanisms, Overlap, and Distinctions, damaged mitochondria can be removed by mitophagy. However, individual mitochondrial proteins can also be damaged and could underlie several pathological phenotypes.[295] It is therefore paramount that the renewal, or turnover, of proteins within mitochondria be sustained in times of enhanced cellular stress, because failure to maintain normal protein turnover can lead to accumulation of damaged/misfolded proteins and could underlie the pathogenesis of various diseases. Protein turnover has been deemed "a missing dimension" in proteomics,[296] because quantitative proteomic measurements typically involve the

profiling of static protein abundance between different disease states or conditions. Recent advances in protein dynamics methodologies have enabled the simultaneous measurement of individual proteins comprising entire mitochondrial[244,297–299] proteomes. A recent study provided the first assessment of global mitochondrial proteome kinetic signatures in a disease model of cardiac remodeling, which demonstrated that protein turnover rates are under independent control, indicative of diverse regulatory processes driving remodeling of the mitochondria in disease.[297]

Protein turnover measurements rely on the ability to track the rate at which individual proteins are being replaced by de novo synthesized proteins. Several methodologies have been used for these measurements, and all involve the introduction of an isotope precursor into a living system to mark individual proteins and determine their longevity in cells. For an excellent comprehensive review of strategies used to measure protein turnover, including the experimental model, stable isotope label, labeling protocol, relative isotope abundance transition, and calculation of turnover rate, see Claydon and Beynon.[300]

Protein turnover rate is evaluated by tracking the integration or loss of a label into a protein pool. Proteins exhibit a diverse range of half-lives, with housekeeping proteins tending toward longer half-lives and regulatory proteins toward shorter half-lives. Thus, sampling times after initiation of labeling must cover an adequate range of time to accurately model proteins that exhibit both fast and slow rates of turnover. Moreover, the number of sampling time points is directly correlated to the accuracy of the labeling trajectory. An additional consideration for complex organisms is the slow equilibration of the stable isotope label (eg, ^{2}H) in a precursor pool (eg, body water), which is incompletely labeled in the in vivo labeling strategies and requires complex analysis to determine precursor pool enrichment. Although each stable isotope methodology has its strengths and weaknesses, heavy water labeling has distinct advantages for translational research in that at low enrichment levels, it is safe for humans over years,[301] it is easy to maintain constant enrichment levels of ^{2}H in body water after $^{2}H_2O$ intake,[302,303] and it is the most cost-effective stable isotope. Detailed methods and equations underlying computational analysis are outlined in Kim et al,[244] and the automated software, ProTurn,[297] is available at http://www.heartproteome.org/proturn/.

Four recent studies have interrogated protein dynamics in mitochondria in whole animals or humans using oral consumption of either deuterated leucine-labeled protein[298] or drinking water.[244,297,299] Two recent studies investigated mitochondrial protein turnover changes in cardiac pathologies. In a translational study by Lam et al,[297] $^{2}H_2O$ labeling was used to investigate changes in protein dynamics of cardiac proteins in mouse and blood proteins in both mouse and human. Turnover rates were determined for 496 human plasma proteins spanning a 50-fold range of turnover rates from albumin (half-life 18.3 days) to insulin-like growth factor 2 (half-life 8 hours). Mice undergoing cardiac remodeling via chronic isoproterenol infusion were compared with controls, and turnover rates for 2964 mouse proteins were assessed in mouse cardiac mitochondrial and cytosolic fractions and in blood. Rates were highly diverse and ranged from <1 day to >3 weeks (100-fold range). Cytosolic proteins turned over ≈10% per day (average half-life of 6.5 days) and mitochondrial proteins ≈5% per day (average half-life 15 days). Consistent with enhanced protein synthesis occurring in cardiac hypertrophy during remodeling, turnover rates in isoproterenol-treated mice were on average 1.2 times faster than in wild-type hearts, with turnover rate increases detected in 972 proteins (>1.3 fold) and decreases in 216 proteins. In contrast, isoproterenol withdrawal, or reverse remodeling, led to an average 20% decrease in protein turnover rate. Importantly, this study identified proteins that exhibited supernormal elevations or attenuations in protein turnover during cardiac remodeling, which suggests that these proteins might be pathogenic in the cardiac remodeling process. Proteins involved in mitochondrial dynamics showed heterogeneous results in that some mitochondrial dynamics proteins exhibited elevated turnover (MIRO1/2, LONP, and PHB), whereas others were unchanged (MFN1/2 and FIS1). Subunits residing in the same respiratory complex (ETC I through V) displayed widespread changes, which provided insight into proteins that could be rate-limiting factors in complex formation and highlights the important finding that synthesis-degradation cycles of proteins within the same functional group (ie, complexes, organelles) are under independent control. Interestingly, turnover measurements also unveiled markedly enhanced turnover in virtually all glycolytic enzymes (HKI half-life decreased from 16.7 to 9.8 days, GAPDH from 10.8 to 6.7 days, and phosphoglycerate mutase-1 from 12.2 to 6.9 days) in the absence of any changes in static protein abundance, thus providing mechanistic insights to support the alteration in substrate utilization, from fatty acids to glucose, known to occur in cardiac disease. These novel mechanistic insights into cardiac remodeling are masked in measurements of static protein abundance, which highlights the unique biological dimension unveiled by protein turnover measurements. Lastly, a study by Shekar et al[299] used $^{2}H_2O$ labeling in rats to measure turnover in heart failure from 2 cardiac mitochondrial subpopulations, subsarcolemmal mitochondria and interfibrillar mitochondria. This group investigated the hypothesis that mitochondrial protein synthesis (and thus, oxidative capacity) is decreased in transverse aorta constriction–induced heart failure, and interfibrillar mitochondria exhibit more pronounced detriments than subsarcolemmal mitochondria. Results from this study showed an overall decrease in mitochondrial content in interfibrillar mitochondria but not subsarcolemmal mitochondria populations, coordinate with a more pronounced detriment in basal and stimulated respiratory rate in interfibrillar mitochondria.

The physiological and pathophysiological implications gleaned from turnover measurements of individual proteins within functional mitochondrial subproteomes are many. Dynamic equilibria of proteins fluctuate in times of cellular stimulation or altered cellular stress, and protein turnover measurements provide a missing dimension of protein behavior that will enable mechanistic studies on the governance of protein synthesis and degradation. Very little is known regarding the interplay of these processes in tuning the abundance of individual proteins or protein complexes in the cell. Global turnover measurements with individual protein resolution, now possible for all detectable proteins within the

mitochondrial proteome, have indicated that diverse regulatory mechanisms exist in metabolic pathways. Furthermore, it is now understood that protein half-life is an exquisitely regulated cellular parameter that is correlated with phenotype; however, it is mostly disassociated with static protein abundance. Protein dynamics provide a unique understanding of the biological regulation of mitochondrial proteins that, when related to static protein abundance and mRNA expression, can inform on the poorly understood process of protein degradation in basal and diseased conditions.

Mitochondrial protein dynamics measurements have high translational significance with regard to mitochondrial biomarker discovery and treatment of mitochondrial diseases. Mitochondrial diseases are a heterogeneous class of conditions that require sensitive and specific biomarkers for their accurate diagnosis and prognostic assessment. The advantages of using protein turnover measurements, rather than the commonly used protein abundance, for early detection of pathophysiological states has been discussed[304] and clearly demonstrated.[305,306] The sensitivity of protein dynamics is in many ways superior to static protein abundance in that alterations in turnover measurements will likely be more pronounced and could precede alterations in static protein abundance in the pathological progression of diseases. Protein turnover rate is also a critical factor in biomarker discovery (Biomarkers), because markers with lower clearance rates within the systemic circulation would be more robust indicators of disease states. Protein dynamics measurements will also unveil novel disease mechanisms (ie, protein degradation insufficiencies), which will spawn the development of novel therapeutic classes.

Questions underlying the relationship between protein turnover and abundance remain. For example, certain mitochondrial proteins exhibit rapid turnover rates (and thus consume a sizeable amount of ATP for their renewal), with little to no change in static abundance. Observations such as these might begin to challenge the conventional school of thought that the abundance of a protein present in a cell directly correlates with its magnitude of impact in the cell. Although several educated guesses have been put forth regarding questions like these, scientific evidence is lacking.

Signaling by Release of mtDNA and Proteins

As discussed in the section on Mitochondria and Cell Death, mitochondria are involved in the intrinsic pathway of apoptosis, where they release soluble proteins, including cytochrome c, from the intermembrane space into the cytosol to initiate caspase activation.[307] The release of these proteins is a consequence of OMM permeabilization. Other examples of functional molecules released from mitochondria include (1) ROS to activate hypoxic gene expression, ROS-dependent mitogen-activated protein kinase, and damaged macromolecules, including DNA and proteins; and (2) calcium, which participates in calcium cross talk between mitochondria and the plasma membrane and between mitochondria and the endoplasmic-sarcoplasmic reticulum and ATP from apoptotic and necrotic cells as a danger signal.[308] Recently, it has been reported that mtDNA and proteins are involved in innate immunity. In this section, we will focus on the role of mitochondria in inflammation.[309]

The immune system is activated not only by microorganism infection but also by endogenous molecules.[310] The endogenous molecules are separated from immune system sensors by plasma membrane and compartmentalization within the cell. However, endogenous molecules are released into circulation during necrosis or into the cytoplasm during degradation of organelles. These endogenous molecules, which can induce inflammatory responses, are referred to as damaged-associated molecular patterns (DAMPs). Pattern recognition receptors (PRRs) are responsible for sensing the presence of microorganisms by recognizing structures that are conserved among microbial species, which are called pathogen-associated molecular patterns (PAMP). Moreover, PRRs are capable of recognizing DAMP. To date, 4 different classes of PRR families have been identified, including transmembrane proteins such as Toll-like receptors (TLR) and C-type lectin receptors, as well as cytoplasmic sensors such as retinoic acid–inducible gene (RIG)-I-like receptors and NOD-like receptors (NLRs). In addition to PRRs, inflammasomes are multiprotein complexes that contribute to the intracellular identification of potentially harmful substances, bacteria, or viruses. Inflammasomes are composed of 3 major components: a characteristic scaffolding protein, the small adapter molecule ASC, and procaspase-1, which is responsible for activation of proinflammatory cytokines. Those scaffolding proteins include the NLR family, pyrin domain–containing 1 (NLRP1), absent in melanoma 2 (AIM2), NLR family CARD domain–containing protein 4 (NLRC4), RIG-I, and NLRP3, and each protein forms the corresponding inflammasome in concert with ASC and procaspase 1.

Because mitochondria are evolutionary endosymbionts derived from bacteria, they retain many morphological and biochemical features of their bacterial ancestors, including a double membrane, membrane lipid (cardiolipin), unmethylated CpG motifs in mtDNA, absence of histones, and the ability to form *N*-formyl peptides, which are synthesized by the use of separate sets of ribosomal RNAs and tRNAs encoded by the mitochondrial genome.[311] Unmethylated CpG motifs and *N*-formyl peptides are inflammatogenic and mitochondrial DAMPs.

In response to pressure overload, mitochondria are damaged, and damaged mitochondria are degraded by autophagy or mitophagy (Mitophagy and Mitochondrial Autophagy: Mechanisms, Overlap, and Distinctions). Among PRRs, TLR9 senses unmethylated CpG motifs in bacteria and virus. mtDNA is degraded by DNase II in the autolysosome, which is an acidic DNase and localized in the lysosome. When induction of autophagy is insufficient in pressure-overloaded hearts, mtDNA escapes from autophagy-mediated degradation and binds to TLR9 to induce inflammatory responses cell-autonomously in cardiomyocytes, myocarditis, and dilated cardiomyopathy. However, it has been reported that depletion of autophagic proteins promotes cytosolic translocation of mtDNA and caspase-1–dependent cytokines mediated by the NALP3 inflammasome in response to lipopolysaccharide in macrophages.[312] In addition to TLR9, the NALP3 inflammasome might be involved in inflammation in the failing heart. The role of autophagy in innate immunity could be dependent on the cell type.

In the case of myocardial infarction, in which necrosis is a main feature of cell death, mtDNA is released into circulation.

The serum from patients after coronary intervention contains mtDNA.[313] Cellular disruption by trauma releases mitochondrial molecules, including DNA, into circulation to activate neutrophils and cause systemic inflammation.[314] Thus, it is possible that mtDNA in circulation after myocardial infarction could contribute to the inflammatory responses in infarct hearts.

Mitochondrial *N*-formyl peptides are released from degenerating mitochondria upon tissue damage[314,315] and recognized by formyl peptide receptors, which have evolved to mediate phagocyte migration to sites of tissue injury. Although the role of the *N*-formyl peptide–FRP signaling pathway in heart diseases, specifically myocardial infarction, remains to be elucidated, it is possible that the pathway could play a role in inflammation in infarcted hearts.

The newly discovered role of mitochondria as DAMP-containing organelles places mitochondria in a central position as initiators and modulators of sterile inflammation in failing or infarcted hearts. In case of pressure-overloaded hearts, autophagy regulates degradation of mtDNA and resultant inflammation in cardiomyocytes. In infarcted hearts, accompanied by necrosis, mtDNA released into circulation could activate and recruit various inflammatory cells in the lesion.

Mitochondria and Cardiovascular Disease

Mitochondrial Myopathies: Mitochondrial Pathogenesis of Cardiomyopathy

Cardiomyocytes have among the highest concentrations on mitochondria of any human cell. Because of the high mitochondrial ATP demands of the heart, relatively subtle defects in the mitochondrial ATP-generating apparatus, oxidative phosphorylation (OXPHOS), can preferentially affect cardiac function.

If we rearrange the classification of the common diseases based on bioenergetics rather than anatomy, it becomes clear that all of the complex diseases can be envisioned as having the same underlying pathophysiological basis: partial bioenergetics dysfunction. Because the mitochondria are assembled from between 1000 and 2000 nuclear DNA (nDNA) genes plus thousands of copies of the maternally inherited mtDNA genes, and the mitochondria both process the calories in our diet into usable energy and are acutely sensitive to a wide range of toxins, it follows that perturbation of mitochondrial bioenergetics can readily explain the pathogenesis of the full range of common clinical disease symptoms (Figure 2).[2,318]

The nDNA-coded genes relevant to mitochondrial function include the ≈1000 proteins located within the mitochondrion[319] plus all of the genes involved in regulating cellular bioenergetics, including the signal transduction enzymes (AMPK, SIRTUINs, etc); nuclear receptor transcription factors (the peroxisome proliferator-activated receptor [PPAR] family, the heteromeric partners of the PPARPs [RXR], the PPAR-γ coactivator-1-α [PGC-1α] family of coactivators, etc); environmentally regulated transcription factors (hypoxia-inducible factor-1α, nuclear factor-κB, etc), nutrient-sensing systems (PTEN [phosphatase and tensin homolog], TSC [tuberous sclerosis complex], mTOR [mammalian target of rapamycin], etc); regulators of mtDNA biogenesis (replication [POLG and Trinkle], transcription [POLRMT], and translation [mitochondrial ribosomal proteins and elongation factors]); and those chromatin remodeling systems that permit nDNA-mitochondrial gene expression. Because of their high energetic demand, the tissues most commonly affected by partial mitochondrial dysfunction are the heart, brain, muscle, renal, and endocrine systems.[2,320–323]

Although the nDNA codes for the great majority of mitochondrial proteins, the mtDNA codes for the 13 most critical OXPHOS polypeptides plus the 22 tRNAs and 2 ribosomal RNAs for their expression. OXPHOS generates energy by coupling electron transport (complexes I, II, and IV) with ATP synthesis (complex V) through the electrochemical gradient.

Severe mitochondrial diseases can result from homozygous mutations in nDNA-coded mitochondrial genes[321,324,325] or severe mtDNA mutations. Milder mitochondrial disease can result from heterozygous nDNA mutations, from mild mtDNA mutations, or from severe mtDNA mutations that are heteroplasmic, a mixture within the cytoplasm of mutant and normal mtDNA. Milder mtDNA mutations result in symptoms when approximating homoplasmic (pure mutant), whereas more severe mutations can lead to disease when heteroplasmic.

There are 3 classes of clinically relevant mtDNA variants: ancient adaptive variants, recent deleterious mutations, and somatic mutations that accumulate in tissues during development and with age. Ancient adaptive mutations have accumulated along radiating maternal lineages as women migrated out of Africa to populate Eurasia and the Americas. A subset of these mtDNA mutations changed OXPHOS function and human physiology, which permitted descendent populations to adapt to the new environments. mtDNAs harboring these locally beneficial variants became regionally enriched by adaptive selection, and as their descendants acquired additional mutations, a group of related haplotypes developed, known collectively as a haplogroup. Although beneficial in one environment, these variants can be maladaptive in another. Various mtDNA haplogroups have now been correlated with a broad spectrum of diseases, including Alzheimer and Parkinson disease, macular degeneration, psychiatric disorders, stoke, diabetes mellitus, cardiovascular disease, sepsis, asthma, AIDS progression, various forms of cancer, types of athletic performance, and longevity.[2,323,325–327]

Recent deleterious mutations continually arise within modern female lineages. Hundreds of such pathogenic mutations have been identified and are cataloged in the mtDNA database, MITOMAP.[328] An example of a frequently homoplasmic "mild" pathogenic mutation is the common Leber hereditary optic neuropathy (LHON) complex I gene missense mutation, *ND4* nt 11778 G>A (R340H).[325,329,330] Examples of more severe heteroplasmic mtDNA mutations are the $tRNA^{Lys}$ nt 8344 A>G mutations, which can manifest as hearing loss at low heteroplasmy but cardiomyopathy and myoclonic epilepsy and ragged red fiber (MERRF) disease at higher heteroplasmy,[331,332] and the $tRNA^{Leu(UUR)}$ nt 3243A>G mutation, which at 50% to 90% mutant heteroplasmy can manifest as myopathy and cardiomyopathy[333] or stereotypically as mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS),[334] but at 10% to 30% mutant can

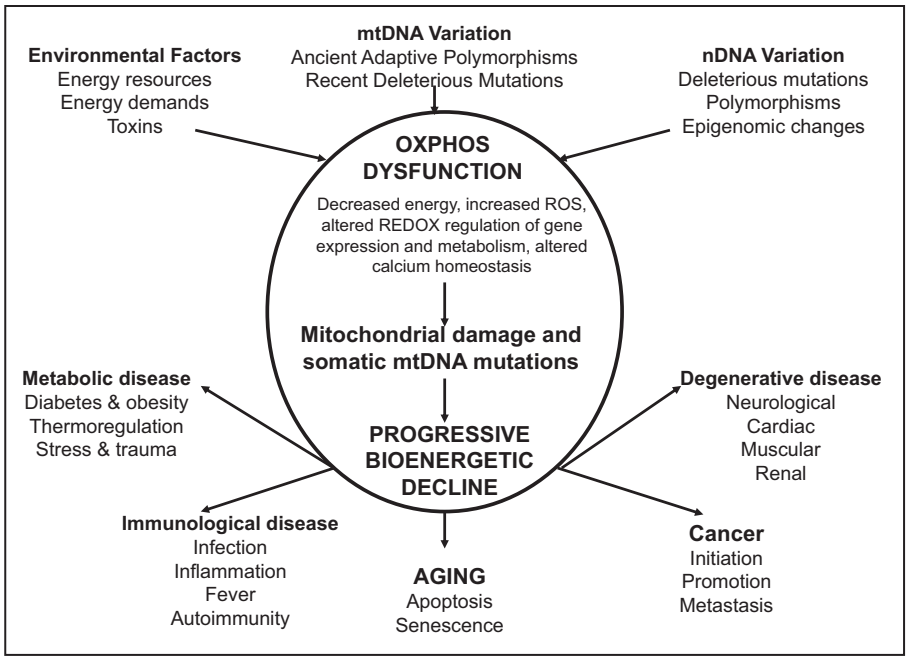

Figure 2. Bioenergetic paradigm for degenerative and metabolic diseases, cancer, and aging. Mitochondrial oxidative phosphorylation (OXPHOS) can be perturbed by nuclear DNA (nDNA) genetic alterations or epigenomic regulation, by mitochondrial DNA (mtDNA) ancient adaptive polymorphisms of recent deleterious mutations, or by variation in the availability of calories and in caloric demands. Alterations in mitochondrial structure and function can impair OXPHOS, which in turn can reduce energy production, alter the cellular redox state, increase production of reactive oxygen species (ROS), deregulate Ca^{2+} homeostasis, and ultimately activate the mitochondrial permeability transition pore, leading to apoptosis. These and other consequences of OXPHOS perturbation can destabilize mtDNA. This results in progressive accumulation of somatic mtDNA mutations and decline of mitochondrial function, which accounts for aging and the delayed-onset and progressive course of degenerative diseases. As energy output declines, the most energetic tissues are preferentially affected, which results in degenerative diseases of the heart, muscle, nervous system, and kidney. Aberrant mitochondrial caloric metabolism also leads to metabolic deregulation, endocrine dysfunction, and symptoms such as diabetes mellitus, obesity, and cardiovascular disease. Energetic failure of apoptosis can result in the release into the bloodstream of mitochondrial antigenic cardiolipin, *N*-formylmethionine polypeptides, and mtDNA (mitochondrial damage–associated molecular patterns [DAMPs][311,314,316,317]) can initiate the inflammatory response, contributing to autoimmune diseases (eg, multiple sclerosis and type 1 diabetes mellitus) and possibly also to the inflammatory component of late-onset degenerative diseases. Finally, cancer cells must manage energy resources to permit rapid replication. Figure reprinted with permission from Wallace.[318] Copyright © 2011, Cold Spring Harbor Laboratory Press.

present as type 1 or type 2 diabetes mellitus[335,336] or autism.[337] Somatic mtDNA mutations accumulate throughout life, progressively eroding mitochondrial function. The rate of accumulation of these mutation can be modulated by nDNA gene variants,[40,210,338,339] which results in degenerative diseases such as Alzheimer disease and Parkinson disease,[340–342] and can be induced by environmental challenges.

A major reason for the complexity of mitochondrial diseases is the reliance of every aspect of cellular function on energy flux. Thus, the energy status of the mitochondrion must be monitored for every functional change in the cell. This is accomplished by all cellular functions being either driven or regulated by mitochondrial high energy or metabolic intermediates. The importance of this mitochondrial signaling to the nuclear-cytosolic epigenome and signal transduction systems has been demonstrated by examining the cellular and transcriptional changes that occur in cells with the same nucleus by different percentages of the $tRNA^{Leu(UUR)}$ nt3243G mutation. This revealed cellular structure and gene transcription changed in discrete phases in response to the progressive increase in percentage of mutant mtDNA, relative to homoplasmic normal mtDNAs, with 20% to 30% 3243G mutant having one transcriptional profile, 50% to 90% mutant a second, and 100% a third. These phase shifts in the transcriptome exactly correspond to the changes in patient phenotypes that are associated with this mutation: 10% to 30% diabetes mellitus and autism, 50% to 90% cardiomyopathy and MELAS, and 100% perinatal lethality.[343]

mtDNA Variation and Cardiomyopathy

Cardiomyopathy has been associated with all 3 classes of mtDNA variants (ancient adaptive mutations, recent maternal mutations, and somatic mutations). Ancient adaptive mutations have been linked to increased risk of metabolic and cardiovascular disease,[344,345] and mice heterozygous for the mitochondrial antioxidant enzyme, MnSOD (*Sod2*), are prone to hypertension.[346]

Recent deleterious mtDNA mutations have repeatedly been linked to cardiomyopathy.[325] Although heteroplasmic mtDNA mutations are commonly accepted as causal for cardiomyopathy, homoplasmic mutations are more difficult to distinguish from ancient adaptive variants that may or may not be contributory. One approach to identify potentially pathological homoplasmic mutations is to first determine the mtDNA haplogroup by use of MITOMAP and MITOMASTER[328] in association with PHYLOTREE,[347] and then to determine whether the putatively deleterious homoplasmic mutation has been observed previously with that haplogroup in the normal population. If not, it is increasingly likely that the homoplasmic

variant could be contributory to the disease.[348] The pathogenicity of the mutation must still be confined by functional tests in cybrids.[349] However, it is also possible that a variant has been observed before but in a different haplogroup context. Such variants could be deleterious on the wrong mtDNA background and thus might contribute to the diseases.[350] Proof that a homoplasmic mtDNA mutation can cause cardiomyopathy comes from the generation of a mouse that harbors an mtDNA missense mutation in the *COI* nt 6598T>C V421A and develops hypertrophic cardiomyopathy.[351]

De novo and somatic mtDNA mutations also cause cardiac disease. Spontaneous single-event heteroplasmic deletions in the mtDNA can cause the Kearns-Sayre syndrome, which frequently presents with cardiac conduction defects and heart block in association with chronic external ophthalmoplegia.[325] The accumulation of a heterogenous array of somatic mtDNA mutations has also been shown to be associated with cardiomyopathy in the hearts of patients who have received heart transplants, as monitored with the common mtDNA 5-kb deletion.[6,352]

nDNA Mitochondrial Gene Mutations and Cardiomyopathy

Cardiomyopathy is one of the primary presentations of boys with Barth Syndrome, which results from mutations in the nDNA-coded X-linked Tafazzin gene required for cardiolipin metabolism.[353] Cardiomyopathy is also the primary symptom of homozygous mutations that inactivate the chromosome 4 heart-muscle-brain isoform of the adenine nucleotide translocator (*ANT1*).[354,355] The ANTs, of which there are 4 in humans, exchange mitochondrial ATP for cytoplasmic ADP across the mitochondrial inner membrane. *ANT1*-inactivating mutations result in mitochondrial cardiomyopathy and myopathy.[356–358] This has been confirmed by the generation of a mouse lacking the *Ant1* gene, which also develops mitochondrial myopathy and hypertrophic cardiomyopathy. The *Ant1*-deficient mouse has a partial defect in cardiac mitochondrial ATP production, because the heart also expresses a second ANT isoform, Ant2. The *Ant1*-deficient mice develop lactic acidosis, mitochondrial ROS production, and a striking increase in the accumulation of cardiac mtDNA somatic mutations.[209,359] The hypertrophic cardiomyopathy of these mice progresses to dilated cardiomyopathy as the mice age.[360] Presumably this is because of the age-related accumulation of somatic mutations that exacerbate the inherited nuclear *Ant1* mutation.

Patients who develop dilated cardiomyopathy associated with myocarditis develop antibodies to ANT.[361,362] These antibodies could be either the initiating or promoting factor in development of the cardiomyopathy. In either case, a mitochondrial bioenergetic defect caused by viral infection or a preexisting mitochondrial genetic defect that impairs mitochondrial energetics could inhibit the energy-demanding apoptotic process, permitting the release of mitochondrial antigens (DAMPs; cardiolipin, mtDNA, *N*-formyl methionine–initiated polypeptides, ANT1, etc)[311,314,316,317] into the bloodstream, where they can initiate a cardiac inflammatory response.

Mutations in many other mitochondria-encoded genes have been reported to cause cardiovascular disease. For example, defects in the mitochondrial matrix protein frataxin are involved in Friedreich's ataxia.[363] Defects in the mitochondrial phosphate transporter have also been associated with cardiac defects.[364]

nDNA-mtDNA Interactions and Cardiomyopathy

The genetic complexity of cardiac diseases is further enhanced by the potential for deleterious interaction of nDNA and mtDNA genetic variants. This pathogenic nuclear-mitochondrial interaction was first demonstrated by the discovery that mutations in the nDNA-coded mtDNA polymerase-γ gene (POLG) can result in autosomal dominant multiple mtDNA deletion syndrome, with the mtDNA damage causing chronic external ophthalmoplegia and (potentially) cardiac symptoms.[325,339] Mitochondrial disease has also been shown to result from the interaction of nDNA-coded partial mitochondrial genetic defects and homoplasmic mtDNA mutations. This was exemplified in a family segregating a missense mutation (G32R) in the X-linked complex I *NDUFA1* gene, which resulted in an ≈30% reduction in complex I, plus a pair of non–haplogroup-associated homoplasmic missense mutations in the mtDNA complex I genes *ND1* 3308T>C (M21T) and *ND5* 12599T>C (M88T). Both the X chromosome and the mtDNA in boys are inherited from the mother, which results in affected boys along the maternal lineage.[365]

The severity of cardiac disease resulting from an nDNA mitochondrial gene defect can also be modulated by the inheritance of an otherwise normal mtDNA haplogroup. In a 13-generation pedigree segregating a frameshift mutation in the *ANT1* gene, multiple homozygous patients were identified with mitochondrial myopathy and cardiomyopathy. However, the severity of the cardiomyopathy varied strikingly, with some individuals progressing to dilated cardiomyopathy that necessitated heart transplantation and others maintaining a relatively stable hypertrophic cardiomyopathy. Sequencing of the mtDNAs from the homozygous *Ant1* frameshift patients revealed that those who progressed to heart transplantation had mtDNA haplogroup U2, whereas those who manifested stable hypertrophic cardiomyopathy had haplogroup H.[358]

This augmentation of deleterious nDNA mutations by mtDNA variants might be a more general phenomenon. Analysis of the mtDNA of cardiomyopathy patients with mutations in the nDNA-coded cardiac contractile apparatus proteins has found that the mtDNA contains potentially contributory mtDNA mutations.[8,366]

Conclusions

The high energetic demands of the heart are primarily met by mitochondrial OXPHOS. Hence, it follows that defects in mitochondrial bioenergetics should preferentially affect the heart. This is proven by the fact that cardiomyopathy is the most obvious phenotypic manifestation in both humans and mice harboring null mutations in the heart-muscle-brain isoform of the ANT. If mutations in nDNA-coded mitochondrial genes can generate cardiomyopathy, it follows that mtDNA mutations that cause partial OXPHOS defects should also contribute to cardiomyopathy. This is supported by the observations that mtDNA haplogroups and de novo mtDNA mutations can augment the deleterious consequences of nDNA mutations. If inherited homoplasmic or heteroplasmic mtDNA mutations can cause cardiomyopathy, the mitochondrial dysfunction that results from

the accumulation of somatic mtDNA mutations could also cause cardiomyopathy. This leads to the conclusion that the age-related accumulation of mtDNA mutations that augment inherited or acquired mitochondrial defects, in addition to the effects of secondary inflammation from the release of mitochondrial DAMPs, could provide a coherent conceptual framework for understanding the progression of multiple forms of cardiomyopathy.

Cardiotoxicity

Anticancer treatments have improved significantly over the past few years; however, despite the improvement in their target effects on cancer cells, anticancer treatments have also been associated with an increase in the incidence of side effects. One of the major side effects of anticancer drugs is their toxicity toward cardiac muscle cells. This cardiotoxicity can manifest at an early stage of therapy (within days) or many years after treatment. Thus, patients undergoing these treatments should be monitored closely. More importantly, patients at high risk should be identified before treatment is started to reduce morbidity from cardiotoxicity. This would require close collaboration between cardiologists and oncologists.

Two forms of cardiac damage have been characterized. One form, typically seen with the use of anthracyclines, is associated with irreversible damage and death of cardiac cells, whereas the second form generally occurs with protein kinase inhibitors and is associated with reversible myocardial dysfunction. These 2 forms are discussed below.

Anthracycline-Related Cardiotoxicity (Type 1)

Anthracyclines have been used for the treatment of various forms of cancer for several decades. They constitute one of the major successes in the field of cancer. For example, in pediatric oncology, the 5-year survival rate has increased from ≈30% in the 1960s to 70% to 80% today,[367,368] and >50% of childhood cancer patients have received anthracyclines.[369] Different statistics have been published on the incidence of anthracycline-mediated cardiotoxicity, ranging from ≈1% to up to 48%.[370] However, a strong correlation exists between the incidence of cardiotoxicity and the dosage of the drug.[371]

Although the effects of anthracyclines on cancer prevention are thought to occur mainly through inhibition of DNA replication, RNA replication, DNA cross-linking, and topoisomerases,[372] their cardiotoxic effects appear to be through distinct mechanisms. The pathophysiology of anthracycline-mediated cardiotoxicity is likely multifactorial, and multiple mechanisms have been proposed. It has been suggested that anthracyclines induce an increase in ROS production.[373,374] According to this model, oxidation of the aglycone portion of doxorubicin results in the formation of a semiquinone radical, which can rapidly revert to its parent compound by using O_2 as an electron acceptor.[375] This futile redox cycle leads to the formation of superoxide, which is converted to H_2O_2 spontaneously or by superoxide dismutase. Subsequently, H_2O_2 may be converted to highly toxic hydroxyl radicals in the presence of heavy metals, such as iron, through the Fenton reaction. In addition, doxorubicin can interact with iron directly to form a doxorubicin-iron complex.[375,376] In addition to production of ROS, other mechanisms including mitochondrial dysfunction and depletion of energy, induction of apoptosis, and changes in topoisomerase IIb activity have been proposed.[377,378]

Although different mechanisms have been proposed for the cardiotoxicity of anthracycline, the mitochondrial effects are likely the major contributor to this disorder. Mitochondrial dysfunction, including mitochondrial swelling, mitochondrial cristae disruption, and accumulation of myelin figures, has been observed after treatment with anthracyclines. There is also evidence that anthracyclines penetrate into the mitochondria (although the mechanism for this transport is not clear). After translocation of anthracyclines into the mitochondria, they can then interact with a number of molecules, including mtDNA, ETC/OXPHOS, mPTP, and mitochondrial iron. Anthracyclines can cause damage by inducing large-scale deletions within mtDNA, form covalent bonds with mtDNA after transformation of the quinone ring to quinone methide, and cause damage to mtDNA indirectly by producing ROS.[379] Anthracyclines can cause damage to ETC and OXPHOS proteins through ROS production or by forming a complex with cardiolipins present in the mitochondrial membrane.[380,381] The latter can also cause an increase in ROS or could lead to cardiolipin dysfunction, which is needed for normal activity of several enzymes in the ETC/OXPHOS pathway. mPTP plays a major role in the regulation of cell death.[382] Anthracyclines have been shown to induce mPTP opening, and cyclosporine A (which inhibits mPTP by binding to cyclophilin D) prevents mitochondrial failure and cell killing.[383] Iron has been known to mediate some of the cardiotoxic effects of anthracyclines; however, iron chelators were shown not to be effective against anthracycline-mediated cardiotoxicity. It has been demonstrated recently that anthracyclines cause mitochondrial iron accumulation and that a reduction in mitochondrial iron is protective against the cardiotoxic effects of anthracyclines.[384]

Drugs for Other Disorders

Several drugs that are currently routinely used for common diseases also have cardiac side effects. For example, it is now known that glitazones can worsen heart failure, and their mechanism of action might be through modulation of the activity of PPAR proteins in the heart.[385] Although PPARs regulate metabolic processes, they might also have an effect on the mitochondria through indirect mechanisms.

Biomarkers

Primary mitochondrial diseases constitute a broad spectrum of disorders that affect multiple tissues and organ systems. It is estimated that mitochondrial diseases affect 1 in 5000 people[386]; however, it is suspected that 1 in 250 might be more accurate,[387] because many cases likely go undiagnosed. Clinical diagnosis of mitochondrial diseases is complicated by diverse phenotypical manifestations, in part caused by variably affected genomes (ie, mitochondrial or nuclear) or organs (eg, muscle, liver) and age of onset. Thus, clinical protocols required for definitive diagnosis are labor intensive and routinely involve invasive procedures with variable success. It would therefore be of great clinical use

to discover biomarkers in accessible biofluids (eg, blood, urine) that specifically inform on primary mitochondrial diseases. Mitochondria-derived biomarkers, including mtDNA (Signaling by Release of mtDNA and Proteins), proteins, and metabolites, have been explored; however, these have exhibited limited specificity, sensitivity, and diagnostic/prognostic accuracy. Current markers can only add weight to the likelihood that a patient might have a primary mitochondrial disorder, and at best may only substantiate a more invasive diagnostic testing regimen.

mtDNA sequencing from intact cells acquired through tissue biopsy (most commonly skeletal muscle) in symptomatic patients constitutes the most definitive current measure for diagnosing mitochondrial disease. However, this method is invasive and is not amenable to routine screening or suitable for high-risk populations. The noninvasive quantification of cell-free mtDNA in plasma has gained substantial interest for diagnosing certain cancers[388,389] and for other clinical scenarios, such as predicting mortality of patients in the intensive care unit,[390] but it has not yet been successfully interrogated for the diagnosis of primary mitochondrial disorders. Mitochondrial proteins and metabolites quantified in accessible biofluids have received limited interest; however, they show great promise as biomarkers of mitochondrial diseases in that they are highly specific and sensitive indicators of underlying metabolic changes.

Improved strategies are warranted for the specific diagnosis of mitochondrial disorders, and biomarkers are most promising in this effort, because disease-specific mitochondria-derived proteins, metabolites, or mtDNA would likely offer superior sensitivity and specificity in diagnosis and risk assessment and could be accurately and consistently measured across all clinical settings. An overview of mitochondria-derived markers (mtDNA, proteins, and metabolites) that have been the subject of intense investigation follows.

Blood and urine are readily accessible biofluids acquired with minimal invasiveness. Blood can be subjected to centrifugation to separate aqueous plasma or serum from blood cells. Mitochondria-derived biomarkers found in plasma or serum are cell-free circulating (cfc)-mtDNA, metabolites, and proteins. Thrombocytes and leukocytes, but not erythrocytes, contain mitochondria, and thus, mtDNA and proteins can be obtained from intact blood cells in the more dense fraction. However, because mitochondrial genomes are heteroplasmic, and primary mitochondrial diseases can affect various organs, disease markers will likely be tissue derived rather than blood cell derived; thus, cell-free markers in plasma likely represent a more relevant and unbiased assessment of mitochondrial disorders. Urine would also provide an unbiased source of metabolites. cfc-mtDNA in plasma and serum has been a subject of intense interest as a biomarker of disease since the first published study showing a mutation in cfc-mtDNA in patients with type 2 diabetes mellitus.[391] mtDNA is a 16.5-kbp circular strand of DNA, and hundreds to thousands of copies of mtDNA can be found in a single cell. Although the precise physiology is unclear, it is thought that cfc-mtDNA enters the bloodstream through cell necrosis, apoptosis, or active secretion.[392] cfc-mtDNA in plasma exists in both particle-associated and free forms, and methodology to measure amounts in whole blood are exquisitely dependent on the preparatory protocols used (eg, size exclusion filtration), which indicates that cfc-mtDNA exists in various conformations.[393] mtDNA can be reliably assayed by a quantitative real-time polymerase chain reaction approach, which exhibits a dynamic range of 5 orders of magnitude and a sensitivity of detection down to 1 copy of mtDNA.[393] Thus, mtDNA possesses favorable physical characteristics for a good candidate biomarker of various physiological states. However, although cfc-mtDNA has shown promise for early detection and tumor classification in oncology patients,[388,389] its role in diagnosis and prognosis of mitochondrial diseases remains to be determined.

Despite the sophisticated and sensitive technologies we have available to measure mitochondria-derived biomarkers, no noninvasive biomarkers for mitochondrial disorders have materialized from the discovery phase to the clinic. One of the most prominent reasons for this is that studies aimed at biomarker discovery have been poorly designed, having little clinical translational potential.[394] Plasma biomarker studies should ideally proceed through an evolution of systematic tests that appropriately credential markers for clinical use (eg, Addona et al[395]). A gap exists between biomarker discovery studies, in which thousands of biomarkers exhibit differential profiles in case versus control subjects, and clinical tests, for which assays are optimized and standardized for rapid and efficient measurement in the clinic. Within this gap is the narrowing and prioritizing of likely biomarker candidates, followed by rigorous steps of validation. Despite the overall lack of clinically available biomarkers, there are promising candidates that have surfaced in recent studies that, with appropriate validation strategies in large clinical cohorts, could add value or replace current clinical markers and diagnostic or prognostic regimens. A study by Suomalainen et al[396] found that serum fibroblast growth factor 21 (FGF-21) was a sensitive and specific marker for primary muscle-manifesting respiratory chain deficiencies in adults and children. This study included 67 patients with mitochondrial diseases diagnosed by muscular biopsy and DNA analysis, 34 control subjects with non-mitochondrial neurological disease, and 74 healthy control subjects. FGF-21 had superior diagnostic accuracy to conventionally used, more nonspecific mitochondrial disease indicators, including lactate, pyruvate, lactate-to-pyruvate ratio, and CK. A later prospective study supported these results and indicated that FGF-21 is a superiorly sensitive biomarker for diagnosing both mtDNA and nDNA-encoded mitochondrial diseases.[397] Taken together, these results showed that FGF-21 might provide a first-tier, noninvasive biomarker for diagnosing mitochondrial diseases that could diminish the need for invasive muscular biopsies. Rigorous validation using large, well-characterized patient cohorts is warranted to verify the utility of FGF-21 in the clinic. Another recent study by Enns et al[398] examined GSH and GSSH protein levels in whole blood from 58 control subjects and 59 patients with primary mitochondrial disease of varying causes, including Leigh syndrome, ETC abnormalities, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes, mtDNA deletion syndrome, and mtDNA depletion syndrome. Quantitative mass spectrometric analysis determined that in patients with

mitochondrial disease, redox status was significantly more oxidized (lower GSH and higher GSSH), showing the greatest change in patients who were hospitalized with metabolic crisis. Hence, GSH and GSSH might be clinically useful biomarkers for all primary mitochondrial disease subtypes, and should be rigorously evaluated in clinical trials to demarcate clinical prognosis and patient response to redox-modulating treatments.

In conclusion, the low diagnostic and prognostic accuracy of primary mitochondrial diseases warrants intense research on mitochondrial biomarkers. The proper experimental design utilizing cutting edge "omics" technologies will undoubtedly propel this area of research in fields of study and is the best response to the challenge of mitochondrial biomarker realization.

Summary and Recommendations

The data in the literature show that mitochondria play a key role in cardiovascular disease, specifically in the response to myocardial ischemia and the transition to heart failure. The accumulation of defects in mitochondrial electron transport, ion transport, metabolism, redox regulation, and mitochondrial quality control leads to a feed-forward cycle of further acquired defects. Ultimately, the mitochondria can no longer meet the high energetic demands of the cardiac cell, and this, coupled with an increase in activation of cell death pathways, leads to the death of the myocytes. The role of mitochondrial defects in heart failure and other cardiovascular diseases needs to be considered and evaluated.

There are a number of gaps in our understanding that need to be addressed in future studies. We need a better understanding of the effects of PTMs on protein function. In recent years, we have made great strides in defining mitochondrial PTM, but the consequences of the modification is poorly understood in many cases. We need additional information on the mechanisms by which mitochondria regulate apoptosis and necroptosis. We need to define the mPTP. The F_1F_0-ATPase has been proposed as the pore; this need to be verified by other groups, and the mechanism of its regulation needs to be defined. We need to define the redox-sensitive component of this pore. We need to more completely define the mechanisms that regulate mitochondrial dynamics and elucidate how this regulates cell function and metabolism. We also need additional information on the mechanisms that regulate mitochondrial turnover at the level of mitochondrial proteins and the organelle. What are the signals for turnover of mitochondrial proteins, and how is this accomplished?

Additionally, the interrelationship between mitochondria and other intracellular compartments (endoplasmic reticulum, lysosomes, etc) and intracellular structures (mitochondria-associated membranes) in the regulation of mitochondrial function and overall cellular homeostasis is increasingly being recognized.[399–401] An understanding of how mitochondrial function and pathophysiology integrate within this more complex intracellular environment will also be necessary to enable the modulation of quality-control programs to sustain cardiomyocyte homeostasis and stress resistance.

Disclosures

Writing Group Disclosures

Writing Group Member	Employment	Research Grant	Other Research Support	Speakers' Bureau/ Honoraria	Expert Witness	Ownership Interest	Consultant/ Advisory Board	Other
Elizabeth Murphy	National Heart, Lung, and Blood Institute	None	None	None	None	None	None	None
Hossein Ardehali	Northwestern University	None	None	None	None	None	None	None
Robert S. Balaban	National Heart, Lung, and Blood Institute	None	None	None	None	None	None	None
Fabio DiLisa	University of Padova	None	None	None	None	None	None	None
Gerald W. Dorn II	Washington University School of Medicine	NIH*	None	None	None	None	None	None
Richard N. Kitsis	Albert Einstein College of Medicine	NIH†; AHA†; Fondation LeDucq†	None	None	None	None	Neuprotect*	None
Kinya Otsu	King's College London	British Heart Foundation†	None	None	None	None	None	None
Peipei Ping	UCLA School of Medicine	None	None	None	None	None	None	None
Rosario Rizzuto	University of Padua	None	None	None	None	None	None	None

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Writing Group Disclosures (Continued)

Writing Group Member	Employment	Research Grant	Other Research Support	Speakers' Bureau/ Honoraria	Expert Witness	Ownership Interest	Consultant/ Advisory Board	Other
Michael N. Sack	National Heart, Lung, and Blood Institute	None	None	None	None	None	None	None
Douglas Wallace	Children's Hospital of Philadelphia	GlaxoSmithKline†	None	Various Symposia*	None	None	None	None
Richard J. Youle	National Institute of Neurological Disorders and Stroke	None	None	None	None	JNPJ†; GE†	None	None

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*Modest.

†Significant.

Reviewer Disclosures

Reviewer	Employment	Research Grant	Other Research Support	Speakers' Bureau/ Honoraria	Expert Witness	Ownership Interest	Consultant/ Advisory Board	Other
Christopher Baines	University of Missouri	NIH (R01 that is studying the role of mitochondria in cardiac disease)†	None	None	None	None	None	None
Lorrie Kirshenbaum	University of Manitoba (Canada)	None	None	None	None	None	None	None
Tibor Kristian	University of Maryland Baltimore	VA Merit (determine the role of cell-type specific mitochondrial dysfunction in mechanisms of acute brain injury)*	None	None	None	None	None	None
Yibin Wang	University of California, Los Angeles	None	None	None	None	None	None	None

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*Modest.

†Significant.

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on behalf of the American Heart Association Council on Basic Cardiovascular Sciences, Council on Clinical Cardiology, and Council on Functional Genomics and Translational Biology

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