J Mol Cell Cardiol **33**, 1065–1089 (2001)
doi:10.1006/jmcc.2001.1378, available online at http://www.idealibrary.com on IDEAL®

Review Article

Mitochondrial Dysfunction in Cardiac Disease: Ischemia–Reperfusion, Aging, and Heart Failure

Edward J. Lesnefsky[1,5], Shadi Moghaddas[1], Bernard Tandler[6], Janos Kerner[4] and Charles L. Hoppel[2,3,5]

Departments of [1]Divisions of Cardiology and [2]Clinical Pharmacology and Departments of [3]Pharmacology and [4]Nutrition, [5]Case Western Reserve University and Geriatric Research, Education and Clinical Center, Louis Stokes Veterans Affairs Medical Center, Cleveland, Ohio, 44106 and the [6]Institute of Environmental and Human Health, Texas Tech University, Lubbock, TX 79409, USA

(Received 21 February 2001, accepted in revised form 1 March 2001)

E. J. Lesnefsky, S. Moghaddas, B. Tandler, J. Kerner and C. L. Hoppel. Mitochondrial Dysfunction in Cardiac Disease: Ischemia–Reperfusion, Aging, and Heart Failure. *Journal of Molecular and Cellular Cardiology* (2001) **33**, 1065–1089. Mitochondria contribute to cardiac dysfunction and myocyte injury via a loss of metabolic capacity and by the production and release of toxic products. This article discusses aspects of mitochondrial structure and metabolism that are pertinent to the role of mitochondria in cardiac disease. Generalized mechanisms of mitochondrial-derived myocyte injury are also discussed, as are the strengths and weaknesses of experimental models used to study the contribution of mitochondria to cardiac injury. Finally, the involvement of mitochondria in the pathogenesis of specific cardiac disease states (ischemia, reperfusion, aging, ischemic preconditioning, and cardiomyopathy) is addressed.

Key Words: Cytochrome; Fatty acid oxidation; Reactive oxygen species; Apoptosis; Preconditioning; Aging; Ischemia; Reperfusion.

Mitochondrial Morphology

Mitochondrial structure (Figs 1–3) provides compartmentalization of mitochondrial metabolism. An outer membrane encapsulates the organelle, while an inner membrane surrounds the central matrix space of the mitochondrion. The outer membrane provides a permeability barrier to cytosol molecules larger than 1500 Da and separates the intermembrane space from the cytosol. The intermembrane space has an ionic composition similar to the cytosol,[1] and contains a distinct group of proteins, including the mobile electron carrier, cytochrome c.[2] The inner mitochondrial membrane consists of regions of inner boundary membrane that are parallel to the outer membrane. Portions of inner membrane invaginate into the matrix as cristae. The cristae include the electron transport chain (ETC), phosphorylation apparatus, and membrane transporters. Pedicles are portions of inner membrane that connect the cristae to the inner boundary membrane (Figs 3 and 4). The inner boundary membrane participates in transport reactions, including the formation of contact sites. The mitochondrial matrix space contains metabolic enzymes, mitochondrial DNA (mtDNA), and RNA.

Contact sites are dynamic structures that involve a fusion of the inner and outer mitochondrial membranes, and are key participants in protein import;[3] energy coupling, with the cytosol via formation of creatine phosphate;[4] and uptake of fatty acids of oxidative metabolism.[5,6] Contact sites in heart mito-

Please address all correspondence to: Edward J. Lesnefsky, M.D., Cardiology Section, Medical Service 111(W), Louis Stokes VA Medical Center, 10701 East Boulevard, Cleveland, OH 44106, USA. E-mail: EXL9@po.cwru.edu

0022–2828/01/061065+25 $35.00/0

Figure 1 Electron micrograph of isolated hamster cardiac mitochondria showing the multiplicity of cristae still in their native configuration and inner and outer mitochondrial membranes. × 28 000.

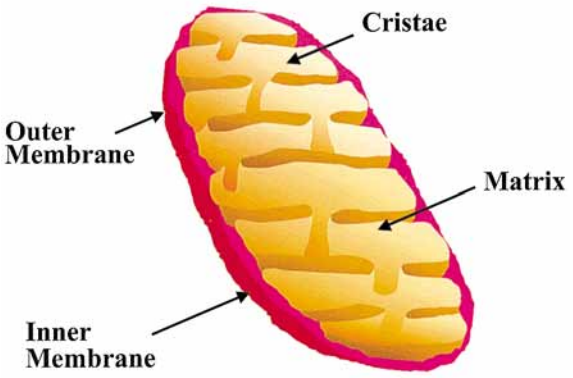

Figure 2 Classical view of an isolated mitochondrion with major structures identified.

chondria are composed of porin in the outer membrane, the octameric form of creatine kinase in the intermembrane space, and the inner membrane adenine nucleotide translocase, facilitating energy flux

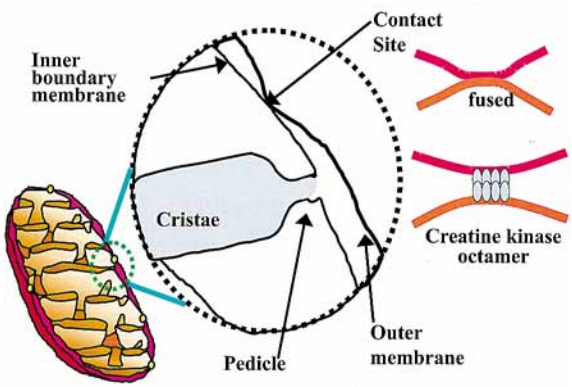

Figure 3 Schematic drawing of a mitochondrion showing contact sites. Contact sites consist of a fusion of the inner and outer membranes and contain octameric creatine kinase. The inner mitochondrial membrane is subdivided into inner boundary membrane, pedicles and cristae. See text for details.

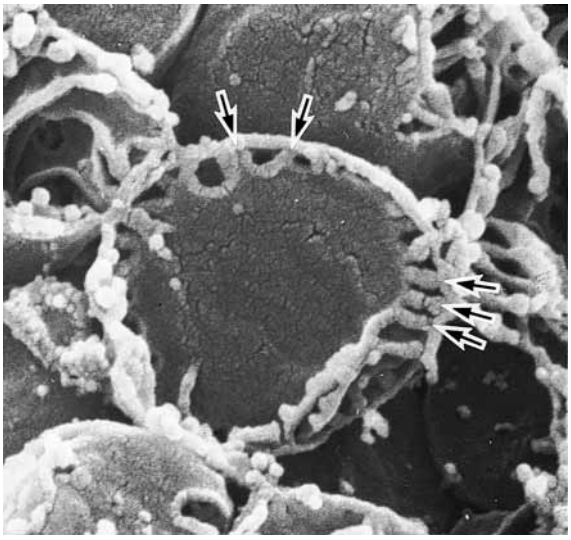

Figure 4 Scanning electron micrograph of a mitochondrion within an oncocyte of the human submandibular gland. This sample was prepared by macerating the tissue for a long period in osmium tetroxide, which removes all soluble components, but which leaves membranes intact. A crista is seen en face; it is connected to the boundary membrane by numerous pedicles, several of which are indicated by arrows. × 35 000. (Micrograph courtesy of Alessandro Riva.)

from the mitochondria to the cytosol.[4,7] A potential role for contact sites in the mitochondrial uptake of long-chain fatty acids has been shown in rat liver mitochondria,[5,6,8,9] and a similar role has been proposed in heart mitochondria.[10] The number of contact sites varies during oxidative metabolism, with an increased number of contact sites present in actively respiring mitochondria.[11]

Mitochondria in Cell Physiology and Metabolism

Regulation of mitochondrial substrate selection

The principal substrates for mitochondrial oxidation are carbohydrates and fats. Carbohydrate metabolism generates pyruvate for mitochondrial uptake. Triglyceride hydrolysis or myocyte uptake of fatty acids provides acyl-groups for mitochondrial activation and uptake. Mitochondrial substrate selection is tightly controlled in response to exogenous substrate availability and the (patho)physiologic state of the myocyte.[12–16] Pyruvate metabolism requires uptake by the pyruvate transporter followed by oxidation by pyruvate dehydrogenase (PDH) to acetyl-CoA. The enzymes involved in fatty acid activation and transport are located in the outer membrane and contact sites.[12] Fatty acid oxidation requires activation of fatty acids by long-chain fatty acid CoA synthetase, followed by formation of acylcarnitines by carnitine palmitoyltransferase-I (CPT-I).[12] The reciprocal regulation of carbohydrate and fatty acid oxidation is controlled at PDH and CPT-I, enzymes that catalyze the committed steps for the oxidation of pyruvate and fatty acids, respectively (Fig. 5).

The activity of PDH is regulated by phosphorylation of the α-subunit of E1 of PDH by PDH kinase, inactivating PDH. Dephosphorylation by PDH phosphatase activates PDH.[17] In heart, PDH kinase isoform 4 predominates, and is rapidly induced by starvation and diabetes, leading to decreased PDH activity. Isoform 4 is relatively insensitive to suppression by dichloroacetate and pyruvate.[18] Fatty acids may directly decrease PDH activity via activation of peroxisome proliferator-activated receptor-α, leading to increased isoform 4 expression in skeletal muscle.[19] Whether this also is the case in heart is not known.

PDH kinase activity is directly inhibited by pyruvate, leading to increased PDH activity. High rates of fatty acid oxidation inhibit PDH via elevated mitochondrial contents of acetyl-CoA and NADH, which activate PDH kinase. In addition, end products of fatty acid oxidation also have a direct inhibitory effect on PDH.[20] The activity of PDH phosphatase is increased by Ca^{2+} and Mg^{2+}, leading to increased PDH activity.[21]

Increased oxidation of pyruvate from carbohydrates inhibits fatty acid oxidation by increasing the content of malonyl-CoA, leading to inhibition of CPT-I. The molecular basis of inhibition is the extreme sensitivity of the muscle isoform of CPT-I, the predominant isoform in heart, to malonyl-CoA ($K_i \cong 0.1$ μM) (Fig. 5).[22] Malonyl-CoA has been proposed as a key component in the regulation of fuel selection by mitochondria. Although this hypothesis is supported by experimental data, unanswered questions remain. First, the mechanism that distinguishes between acetyl-CoA derived from the oxidation of either pyruvate or fatty acids remains unclear. Are there separate mitochondrial acetyl-CoA pools? One pool derived from pyruvate oxidation would be amenable to export to the cytosol for malonyl-CoA synthesis, whereas the other pool, derived from β-oxidation of fatty acids, is restricted to the matrix. Second, the reported malonyl-CoA content in the heart (approx. 2–5 μM)[23,24] is several-fold higher than the K_i of malonyl-CoA for CPT-I. Yet fatty acids still are oxidized, providing the main fuel for the heart under resting conditions. To resolve this apparent conundrum, a compartmentalization of malonyl-CoA has been proposed, but not experimentally verified. Indirect evidence for malonyl-CoA compartmentalization recently has been obtained by demonstrating an equal subcellular distribution of malonyl-CoA decarboxylase, representing the main route of malonyl-CoA disposal, in mitochondrial and extramitochondrial compartments in heart (Kerner and Hoppel, unpublished data). Third, changes in the flux of fatty acid oxidation do not always result in appropriate directional changes in tissue malonyl-CoA concentrations, suggesting the presence of additional regulatory mechanisms. Cytoskeletal proteins in liver interact with mitochondria and modulate CPT-I activity.[25] Palmitoyl-CoA, the substrate for CPT-I, also competes with malonyl-CoA for binding at the regulatory site. Thus, increasing tissue concentrations of palmitoyl-CoA should dampen CPT-I inhibition at any given malonyl-CoA concentration, leading to an increased flux through fatty acid oxidation as the ratio of palmitoyl-CoA: malonyl-CoA increases. Experimental support for this regulatory mechanism recently has been presented.[15] An increased palmitoyl-CoA:malonyl-CoA ratio due to increased uptake and activation of fatty acids and decreased malonyl-CoA content could also explain the high rates of fatty acid oxidation[15] observed during reperfusion.[26]

Oxidative mitochondrial metabolism

The matrix space contains the enzymes of the tricarboxylic acid (TCA) cycle, biosynthetic enzymes, and antioxidant defense enzymes. TCA cycle oxidation of acetyl-CoA generates NADH and

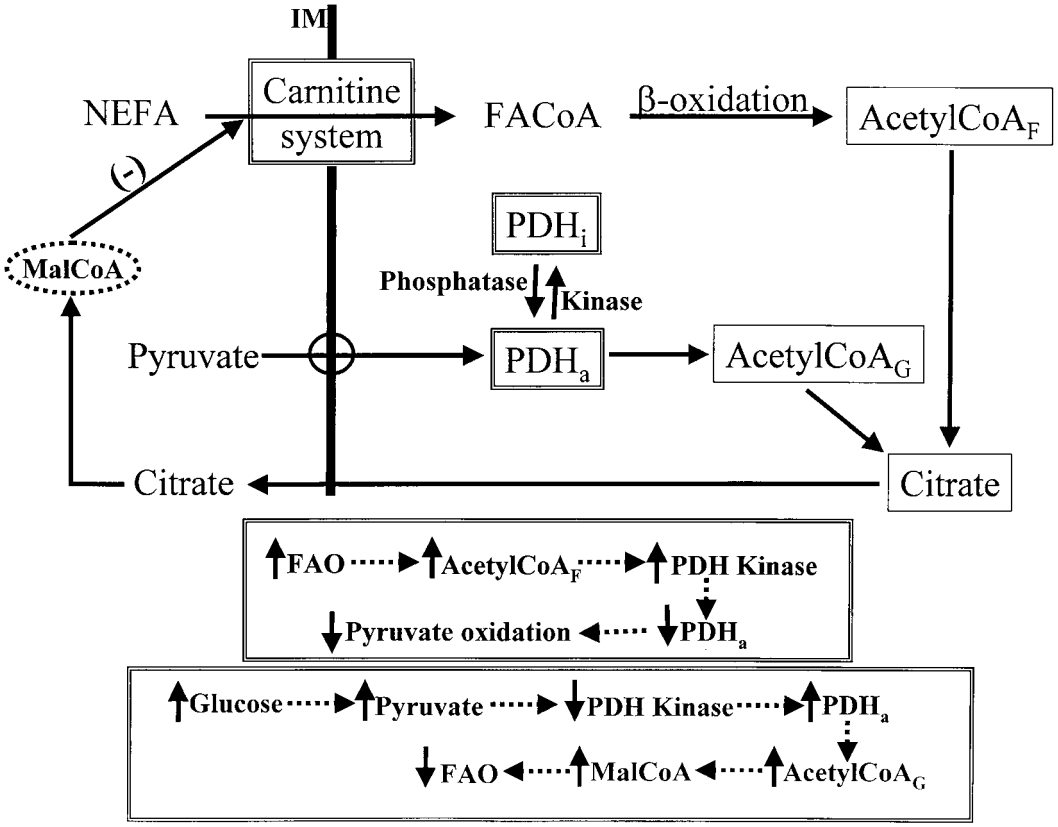

Figure 5 CPT-I, the first enzyme of the mitochondrial carnitine system, is localized in the outer membrane with the malonyl-CoA binding site exposed to the cytosol. Intramitochondrial acetyl-CoA must be converted to cytosolic malonyl-CoA to inhibit fatty acid oxidation. Increased pyruvate decarboxylation leads to increased mitochondrial acetyl-CoA$_G$ and to incresead citrate formation. Citrate is transported into the cytosol and reconverted by citrate lyase to acetyl-CoA, which is carboxylated to malonyl-CoA by acetyl-CoA carboxylase. (An alternative mechanism of mitochondrial acetyl-CoA export, not depicted here, is via acetylcarnitine and carnitine–acylcarnitine translocase as described in the text.) Conversely, increased fatty acid oxidation leads to an increased acetyl-CoA (acetyl-CoA$_F$) and NADH formation. Activation of PDH-kinase and direct inhibition of PDH$_a$ leads to the decrease in mitochondrial pyruvate oxidation.

$FADH_2$ for oxidation by the ETC (Fig. 6). The ETC consists of four multi-subunit enzyme complexes, and the mobile electron carriers, coenzyme Q (inner membrane) and cytochrome c (intermembrane space), that pass electrons sequentially from high (NADH or $FADH_2$) to low (molecular oxygen) redox potential (Fig. 6). The multi-subunit complexes of the ETC diffuse individually in the semi-fluid inner membrane cristae. Electron transfer occurs via productive collisions.[2] Recently, the organization of complexes into a larger supercomplex, a respirasome, has been proposed.[27] The subunit composition, 3-dimensional structure, and structure–function relationships of the ETC complexes [complex I,[28] complex II,[29] complex III,[30] and complex IV (cytochrome oxidase)[31]] have been extensively studied and reviewed.

The inner membrane is impermeable to ions and small molecules as predicted by the chemiosmotic theory of Mitchell.[32] Active transport of hydrogen ions from the matrix side to the cytosolic side of the inner membrane stores energy released during electron transport. The inner membrane contains transporters that regulate the uptake of small molecules and ions into the matrix.

The phosphorylation apparatus consists of the complex V inner membrane ATPase, phosphate transporter, and adenine nucleotide translocase.[33] Inorganic phosphate and ADP are translocated across the inner mitochondrial membrane. Complex V provides a path for protons to flow down the electrochemical gradient from cytosol to the matrix side of the inner membrane, harnessing the energy to phosphorylate ADP.[34] Mitochondrial oxidation is coupled in part to myocyte energy demand through this process. A decreased availability of ADP slows the rate of phosphorylation (state 4 respiration), leads to an enhanced electrochemical proton potential across the inner membrane, and slows the rate of electron transport to oxygen. An increase in

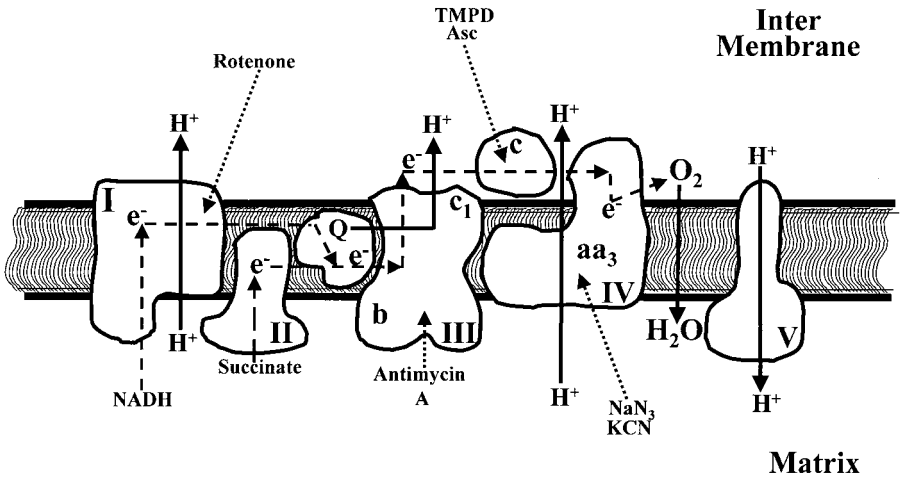

Figure 6. The entry of reducing equivalents from NADH, succinate, durohydroquinone (DHQ), and TMPD-ascorbate (TMPD-asc) into the electron transport chain is shown. Glutamate generates NADH via glutamate dehydrogenase that is oxidized by complex I with electron flow via coenzyme Q (Q), complex III, cytochrome c, and cytochrome oxidase (complex IV). Succinate is oxidized by complex II. DHQ is oxidized by complex III. TMPD-ascorbate reduces cytochrome c that is oxidized by complex IV. Complex III contains cytochromes b and c_1. Complex IV contains cytochrome aa_3. Rotenone and amytal inhibit complex I, myxothiazole and antimycin A inhibit complex III, and azide and potassium cyanide (KCN) inhibit complex IV.

ADP content secondary to increased energy demand will increase phosphorylation and utilization of the proton gradient by complex V, stimulating electron transport and oxygen consumption (state 3 respiration).

Uncoupling of the rate of respiration from ADP availability occurs due to physiologic control and in pathologic states. Complex IV permits continued electron transfer in the absence of proton translocation.[35] Continued electron transfer prevents the accumulation of electrons on upstream redox centers, and decreases the formation of reactive oxygen species.[35,36] Uncoupled respiration occurs in thermogenesis due to the action of uncoupling protein.[37] Uncoupling in pathologic states can occur as a result of inappropriate activation or induction of uncoupling proteins[38] or damage to either the integrity of the inner membrane or of complex V. Damage to complex V or the inner membrane allows back diffusion of protons, dissipates the electrochemical gradient "de-energizing" mitochondria, and blunts phosphorylation.

Production of reactive oxygen species by mitochondria and mitochondrial antioxidant defense

During normal respiration, approximately 2–4% of electron flow through the ETC results in only partial reduction of O_2, generating superoxide ($\bullet O_2^-$).[39,40] The sites of "electron leak" during normal respiration provide insight into the likely sources of increased oxyradical production in pathologic states. The NADH dehydrogenase in complex I and the ubiquinone–cytochrome b area of complex III generate $\bullet O_2^-$ during normal respiration.[39–42] In contrast, although complex IV contains intermediates corresponding to the three oxyradicals: $\bullet O_2^-$, hydrogen peroxide (H_2O_2), and the reactive hydroxyl radical ($\bullet$OH), these intermediates are tightly bound and chemically "disguised", preventing production of reactive oxygen species by complex IV.[43] Conditions that support the accumulation of electrons at redox centers susceptible to electron leak enhance the production of oxyradicals.[39,41,42,44,45] An increased reduction of the ETC favoring electron leak occurs during state 4 respiration[44,45] and ischemia[46,47] when contents of NADH and $FADH_2$ are increased and stimulants of electron transport, ADP and/or O_2, are decreased. Based on the localization of the sites of the production of oxyradicals within complex I, $\bullet O_2^-$ formation should occur on the matrix side of the inner membrane. Complex III can release oxyradicals to either the matrix[41,42] or intermembrane space[48] compartments. The continuous "low level" oxyradical generation during normal respiration increases the oxidation of mitochondrial proteins,[49] lipids,[50] and DNA.[51] More extensive oxidative damage is prevented by mitochondrial antioxidant enzyme systems.[52–57]

Manganese-containing mitochondrial superoxide dismutase, located in the matrix, eliminates $\bullet O_2^-$ by catalyzing dismutation to H_2O_2. H_2O_2 is in-

activated by either catalase[52] or by the glutathione redox system consisting of reduced glutathione as the cofactor for glutathione peroxidase and glutathione reductase.[53–55] The relative contributions of catalase[52] and glutathione peroxidase[53] in H_2O_2 degradation in cardiac mitochondria remain unclear. These enzymes serve to minimize the accumulation of $\bullet O_2^-$ and H_2O_2, which in the presence of the redox-active transition metals, copper and iron, form the very reactive and damaging ●OH, for which no antioxidant enzyme system exists.[55]

Enzyme systems also exist in mitochondria that repair oxidative damage. Phospholipid hydroperoxide glutathione peroxidase is a selenium-containing enzyme that directly reduces peroxidized acyl-groups in phospholipids.[56] Repair of oxidized protein sulfhydryl groups occurs via thioredoxin and thioltransferase enzymes.[58,59] Phospholipid hydroperoxide glutathione peroxidase has both cytosolic and mitochondrial isoforms.[56]

Mitochondrial DNA (mtDNA)

mtDNA consists of circular double-stranded DNA located in the matrix in close proximity to the inner membrane.[60] Propinquity to the sites of the generation of reactive oxygen species suggests that mtDNA is highly susceptible to oxidative damage.[51] Oxidative modification of bases in mtDNA is observed more frequently than in nuclear DNA.[51,60] Recent work has shown that mitochondria contain DNA repair enzymes, including those for base excision repair.[62,63] The susceptibility orf mtDNA to oxidative damage is probably less than previously thought.

Nearly all of the mitochondria in the zygote are derived from the oocyte.[68] Thus, mitochondrial cytopathies secondary to mtDNA mutations display a maternal inheritance.[60] mtDNA also is subject to somatic mutations during the life of the cell, important in the post-mitotic cardiac myocyte.[60,62] The frequency of mutations in mtDNA in cardiac myocytes increases with age in both experimental animals and humans.[62,63] A frequently observed mutation is a deletion of 4977 bp.[60,63,64] Disease states, including chronic myocardial ischemia, increase the frequency of mutations in mtDNA.[64] However, the link between a genotype of mtDNA mutation and a phenotype of cellular dysfunction has proved more elusive in the case of acquired mutations. Due to heteroplasmy (many copies of mtDNA per cell), approximately 80% of mitochondria must be affected in order to result in loss of mitochondrial function.[65–67] While it initially seems unlikely that 80% of the mitochondria in any cardiac myocyte would sustain the same stochastic somatic mutation and induce a phenotype, several factors make the occurrence more likely. First, mtDNA mutations appear to segregate, leading to a "mosaic" pattern of myocyte involvement.[65] Thus, rather than each myocyte consisting of 20% impaired mitochondria, 20% of the cells may have nearly 100% of mitochondria affected. Mosaic patterns of loss of complex IV occur in the aging heart.[68] Second, mitochondria containing mutations can segregate along the length of the myocyte.[66] Additionally, while currently not well understood, certain mutations, such as the "large deletion", appear to occur more frequently than other deletions, suggesting that deletion occurrence may not be a completely stochastic process.[63,64] Finally, while "loss of function" may require a substantial number of affected mitochondria, acquisition of pathologic function, including cell damage secondary to enhanced production of oxyradicals or release of cytochrome c, may require far fewer affected mitochondria per myocyte.

Mitochondrial proteins

Mitochondria contain both nuclear-encoded proteins and proteins encoded by mtDNA. mtDNA encodes for specific subunits of the ETC complexes, most of which are catalytic subunits directly involved in the transfer of electrons. mtDNA encodes for 13 proteins, including seven subunits of the 42 subunit complex I, cytochrome b of the 11 subunit complex III, and subunits I, II, III of 13 subunit complex IV.[60] The mitochondrial genome is transcribed in an "all or none" fashion without transcriptional regulation of individual genes.

The majority of mitochondrial proteins are encoded by the nuclear genome, synthesized on cytosolic ribosomes, and imported into mitochondria. Mitochondrial protein import is highly regulated, with import mechanisms consisting of protein chaperones[69,70] and transmembrane peptide import systems. The transmembrane peptide import complexes translocate proteins into the appropriate mitochondrial space (translocase outer membrane—TOM,[69] translocase inner membrane—TIM[70]) based upon specific mitochondrial targeting sequences in amino terminal portion of the preprotein that are cleaved following import.[69] Direction of proteins to specific mitochondrial compartments contributes to the regulation of mitochondrial metabolism.

Cytochrome c is synthesized as a nuclear-encoded

apoprotein with a mitochondrial targeting sequence.[71] During import into mitochondria, the intermembrane space enzyme, ferrochelatase, inserts the iron into apocytochrome c. Addition of iron is required for successful import into the intermembrane space.[71] The presequence is then cleaved, generating functional cytochrome c.

The role of mitochondrial–nuclear genomic interactions in the proper assembly of ETC complexes, composed of both nuclear and mtDNA-encoded subunits, remains unknown. Subunits present in greater excess than required for assembly of ETC complexes are degraded by matrix proteases.[72] It is currently unclear if ETC complexes, or their damaged subunits, can be replaced in existing mitochondria, or if assembly of electron transport chain complexes occurs only during mitochondrial proliferation. The signals for replacement of defective subunits or complexes, or even the question if mtDNA transcription can drive synthesis of nuclear-encoded subunits required to complete assembly of the ETC complexes remain unknown. If transcription of nuclear-encoded proteins does respond to defects in ETC complexes, this response can provide a sensitive index for the loss of mitochondrial function at the cellular level. These issues have an impact upon the potential for restoration of mitochondrial function in pathologic states. It has been reported recently that heart failure increases transcription of adenine nucleotide translocator-1 and subunits of complex V,[73] suggesting that transcription of nuclear-encoded mitochondrial proteins are regulated in response to changing conditions in the myocyte.

Experimental Approaches to Study Mitochondrial Metabolism

Experimental preparations ranging from the intact heart to structural and functional characterization of individual molecular components of mitochondria are studied. The different experimental systems each have strengths and weaknesses, and provide complementary strategies to elucidate the role of mitochondria in cardiac disease.

Isolated perfused heart

The isolated, perfused heart is used to study the impact of substrate selection on resting cardiac function. Measured oxygen consumption compared with mechanical work yields estimates of cardiac metabolic efficiency.[74] Contractile recovery and the extent of tissue injury during post-ischemic reperfusion varies as a function of the substrate furnished to the heart,[75–78] providing insight into the contributions of altered mitochondrial metabolism to physiology of the intact heart.

Isolated myocytes

Intact, isolated myocytes provide an approach to study *in situ* mitochondrial function, including membrane potential,[79] calcium content,[80] and production of reactive oxygen species.[47] *In situ* studies require the use of fluorescent dyes as indicators of mitochondrial function, usually assessed by confocal laser microscopy. The major strength of this approach is the ability to study mitochondria *in situ*. Studies using fluorescent dyes as endpoints are limited by the specificity of the particular dye and by issues regarding fluorescence quenching, and linearity of response.[81,82] Care must be taken to achieve proper dye concentrations so that mitochondrial, and not plasma membrane, potential is observed.[81,82] With attention to these concerns, studies utilizing intact myocytes have provided important insight into mitochondrial function in pathologic states.

Permeabilized myofibers or myocytes are used to study substrate oxidation by *in situ* mitochondria. The plasma membrane is permeabilized with digitonin or saponin to allow equilibration of cytosol and media. Substrates are added and oxygen consumption measured. With this approach, the interaction of *in situ* mitochondria with cytoskeletal components can be studied, whereas interactions with the cytosol are lost. Due to the presence of non-mitochondrial ATPases, oxidative phosphorylation and coupling of respiration to energy demand cannot be studied in detail.

Isolated mitochondria

Using isolated mitochondria (Fig. 7), oxidative metabolism can be studied in detail, including the coupling of oxidation and phosphorylation. Titration of activity with inhibitors of respiration is used to study the control strength of the different components on the overall rate of oxidative phosphorylation.[83–85]

Cardiac mitochondria exist in two functionally distinct populations, subsarcolemmal mitochondria (SSM) residing beneath the plasma membrane, and interfibrillar mitochondria (IFM) located between

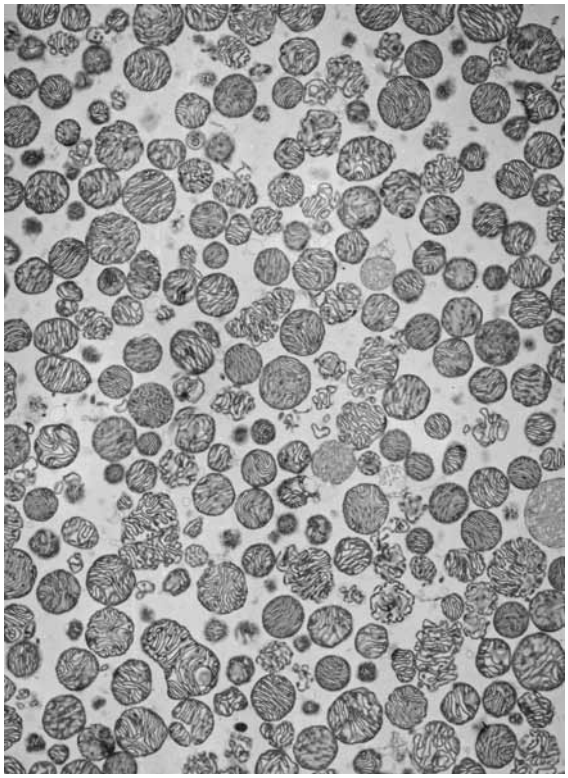

Figure 7 Survey micrograph of a pellet of mitochondria isolated from the rat heart. Note purity of the sample. $\times 4400$.

the myofibrils (Fig. 8).[86–93] ADP-stimulated respiratory rates (state 3) are greater in IFM than in SSM, whereas the coupling of respiration is similar in both populations.[88,89] IFM have an increased content of respiratory cytochromes, and the activity of ETC complexes is greater in IFM than in SSM.[88,89] Differences in respiratory rates and enzyme contents persist following exposure of each population to the methods used to isolate the other population.[89] A distinct structural marker for each population has not been identified. However, the two populations are affected differently in pathologic states, including calcium overload,[90] cardiomyopathy,[91] aging,[92] and ischemia.[87,88,93] Thus, consideration of regional differences in mitochondrial response to disease states is required in order to identify novel mitochondrial defects present in pathophysiologic states.

Mitochondrial components

Mitoplasts are obtained from isolated mitochondria following removal of the outer mitochondrial membrane by digitonin, French press, or osmotic swelling and shrinking. Mitoplasts are useful for the study of processes localized to the inner membrane or to the matrix in a preparation with retained respiration and respiratory coupling.[5,94,95] Mitoplast preparation releases contents of the intermembrane space for isolation and study. Sonication of intact mitochondria followed by sedimentation of the resulting "inside-out" inner membrane vesicles yields submitochondrial particles (SMP).[96] SMP are devoid of matrix contents and lack respiratory control. SMP are useful for studies that require an increased concentration of ETC complexes, including EPR.[97] The lack of antioxidant enzymes makes SMP attractive for the study of the generation of reactive oxygen species from specific sites in the ETC.

Integrative approach to the study of mitochondrial pathophysiology

The polarographic study of oxidative phosphorylation in isolated mitochondria provides an approach to localize defects in the ETC and to identify damage to the phosphorylation apparatus that results in diminished coupling of respiration. Substrates that donate electrons to specific sites in the ETC are used to localize the sites of defects (Fig. 6). Measurement of uncoupled respiration evaluates whether or not damage to the phosphorylation apparatus is the mechanism of decreased oxidative phosphorylation. Exposure of mitochondria to freezing–thawing and hypotonic conditions permeabilizes the inner membrane, removing transport barriers. Measurement of oxygen consumption in permeabilized mitochondria bypasses defects in substrate carriers or dehydrogenases and confirms localization of defects to the ETC. Measurement of enzyme activities of the specific ETC complexes in solubilized mitochondria directly localizes defects in the ETC predicted from the study of intact and permeabilized mitochondria.

Mechanisms of Mitochondrial-derived Tissue Injury

Mitochondria contribute to myocyte injury via loss of physiologic function or acquisition of pathologic function. The physiologic significance of damage to mitochondrial DNA, proteins, or lipids should be established at the level of the mitochondrion, myocyte, and whole heart. Since many mitochondrial enzyme systems are present in excess relative to the amount required to support maximal rates of oxidative phosphorylation in intact mitochondria,

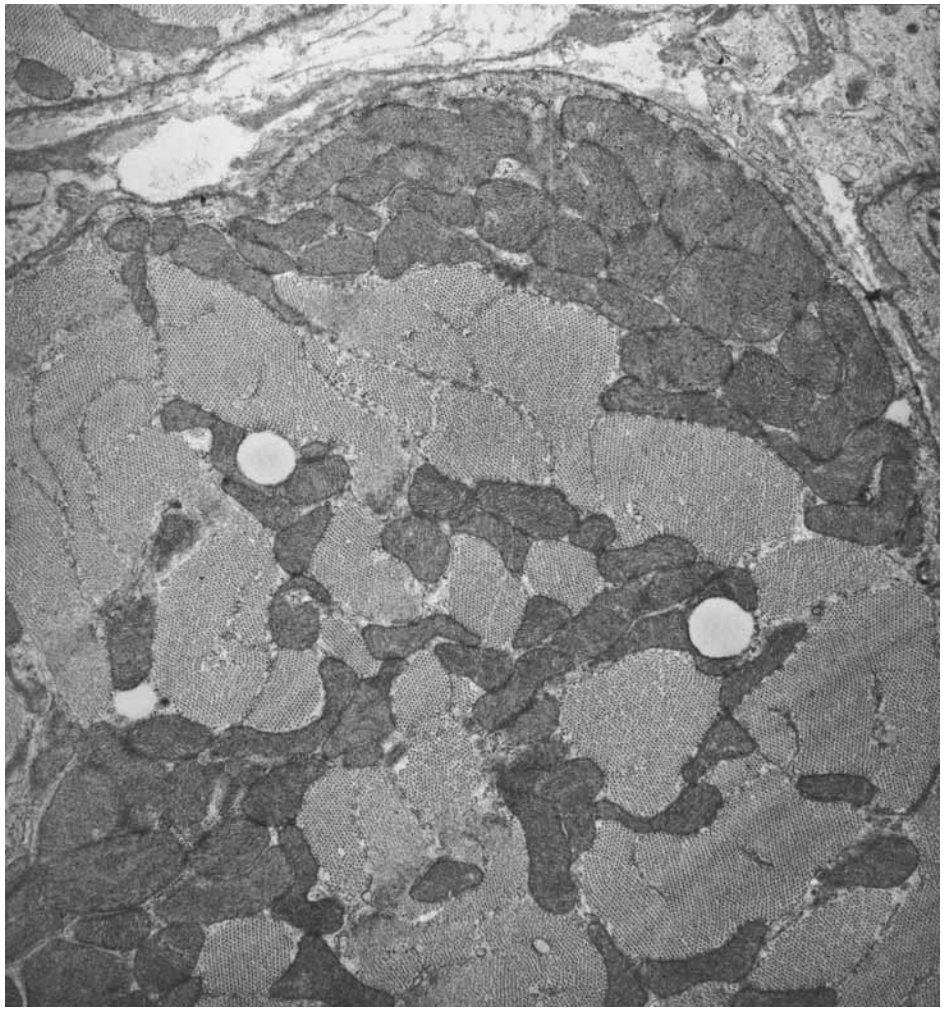

Figure 8 Cardiac mitochondria exist in two functionally distinct populations. Subsarcolemmal mitochondria are situated beneath the plasma membrane (sarcolemma), while interfibrillar mitochondria are ensconced among the myofibrils. Transverse section of a cardiomyocyte in the hamster heart showing a cluster of subsarcolemmal mitochondria at the top of the micrograph and a cluster of interfibrillar mitochondria at the lower left corner. Other interfibrillar mitochondria are scattered among the myofibrils. $\times 7200$.

the physiologic significance of loss of mitochondrial enzyme activity needs to be established. Loss of function secondary to molecular damage is established at the mitochondrial level by the persistence of defects in mitochondrial oxidative physiology under non-maximal, as well as maximal rate conditions. While the use of maximally expressed activity is useful to establish the presence of enzyme defects, mitochondrial respiration *in situ* operates at non-maximal rates.[83–85] Significant defects should also exhibit a greater sensitivity to inhibitor titration,[98] providing evidence for new sites of control of mitochondrial oxidative metabolism secondary to newly acquired partial blocks in oxidative metabolism. Loss of function also is expected to result in the accumulation of substrates that are ordinarily oxidized. Significant loss of mitochondrial function should have an impact on cell physiology via the deleterious accumulation of substrates or the impairment of energy-dependent cellular processes. The loss of mitochondrial function present at the cellular level should translate to loss of physiologic function or to enhanced tissue injury in the intact heart.

Loss of mitochondrial function may be compensated for at a cellular or organ level, thus requiring a comprehensive approach to study the impact of mitochondrial defects. It remains uncertain if all mitochondria are impaired to a similar extent, or if a fraction of mitochondria is severely affected. In the latter case, mitochondria with preserved function may compensate for affected mitochondria. Mitochondria also may be affected on a cell by cell basis, rather than all cells containing a

similar fraction of affected mitochondria. Thus, while mosaic patterns of involvement dramatically impair metabolism in severely affected cells, perhaps even leading to cell death, other myocytes with unimpaired mitochondrial function may compensate for severely affected cells, preserving cardiac function. Development of a disease phenotype thus requires progression until the number of involved myocytes is sufficient to alter cardiac physiology.

The acquisition of pathologic mitochondrial function occurs secondary to the production of compounds not present during normal metabolism; an increase in the content of a deleterious compound present at a low concentration during normal metabolism; or release of a constituent normally sequestered in mitochondria. In contrast to loss of function, gain of pathologic function is likely to require fewer mitochondria to produce deleterious consequences for the myocyte. Functional or anatomic subpopulations of cardiac mitochondria thus can exert harmful effects upon the myocyte. The physiologic relevance of injurious mitochondrial effectors should be demonstrated by the use of interventions, either metabolic or pharmacologic, that negate production of the deleterious metabolite and allay cell and organ dysfunction and tissue injury.

Loss of mitochondrial function

Decrease in production of high energy phosphates

Damage to cardiac mitochondria impairs energy production by several mechanisms. First, direct damage to the ETC leads to decreased rates of substrate oxidation.[87,93,99] Enzyme activities of ETC complexes are present in excess relative to oxidative phosphorylation, so decreases in ETC enzyme activities of 30–50% are needed to lower the rate of oxidative phosphorylation.[83–85] In the baseline state, the sites of greatest control of oxidative phosphorylation are present in complex I, the adenine nucleotide translocase, and complex V.[83–85] These sites require the least inhibition for decreases in the rate of oxidative phosphorylation to become apparent. Damage to specific sites in the ETC introduces additional sites of partial block in electron transport, generating new sites of control of oxidative phosphorylation.

Damage to the phosphorylation apparatus also impairs energy production. Direct damage to complex V decreases the rate of efficiency (coupling) of phosphorylation of ADP.[99] Damage to the inner membrane dissipates the proton gradient, de-energizing mitochondria and blocking phosphorylation. Last, damage to mitochondrial creatine kinase, an oxidatively sensitive enzyme,[100] impairs the creatine shuttle, blunting export of high energy phosphates from mitochondria. Thus, damage to mitochondria at any one of several sites can impair energy production.

Accumulation of metabolic intermediates

Loss of mitochondrial function also may result in the accumulation of compounds toxic to the myocyte. Decreased oxidation of pyruvate leads to lactate production and acidosis observed in ischemia and mitochondrial myopathies. Impaired oxidation of lipid substrates results in accumulation of long-chain acyl-CoA and acylcarnitines.[101–104] These compounds inhibit PDH, block carbohydrate oxidation,[103] accumulate in myocyte membranes,[101] enhance myocyte calcium loading,[104] and lead to arrhythmias by direct detergent action and by activating calcium channels.[101]

Acquisition of pathologic mitochondrial function

Production of reactive oxygen species

Damage to mitochondria enhances the production of reactive oxygen species.[100] Two mechanisms combine to increase oxyradical production. First, decreased flux through the ETC increases the reduction of proximal sites in the ETC, enhancing "electron leak" that forms $\bullet O_2^-$.[44,45] Second, damage to individual ETC complexes directly results in electron leak and oxyradical production. Damage to complex I directly increases the production of oxyradicals.[106–108] Ischemic damage to complex III superimposed upon preexisting aging defects may enhance the production of oxyradicals in the aging heart.[109]

Release of cytochrome c

Release of cytochrome c from mitochondria is a key step leading to programmed cell death.[110–112] Although apoptosis can occur in the absence of cytochrome c release, cytochrome c directly activates downstream effectors when injected into the cytosol in the absence of upstream signals.[113] Following release of cytochrome c, a complex is formed in the cytosol in the presence of APAF-1, deoxyATP, and the cysteine protease caspase-9. This complex then

directly activates downstream effectors of the apoptotic pathway including caspase-3.

Regulation of cytochrome c release by mitochondria currently is being investigated. Release may occur secondary to the onset of mitochondrial permeability transition (MPT).[113] MPT occurs secondary to formation of an inner membrane pore permeable to solutes of less than 1200 Da.[113,114] The onset of MPT leads to loss of mitochondrial membrane potential and to swelling of the matrix space with eventual disruption of mitochondrial membranes and release of cytochrome c.[110,113] This catastrophic event is caused by oxidative damage to mitochondria in concert with mitochondrial calcium overload, conditions frequently observed during ischemia and reperfusion. The "pore" of MPT contains porin and adenine nucleotide translocase, both components of contact sites, and might represent pathologic alteration of a contact site. Under ischemic conditions, there is a transition from the octameric to the dimeric form of creatine kinase.[115] The dimeric form of creatine kinase, porin, and adenine nucleotide translocase exhibit some properties of the MPT pore when incorporated into membrane systems.[116,117]

However, cytochrome c release is highly regulated, and probably occurs following events other than the catastrophic mitochondrial damage observed with MPT in experimental studies. First, MPT itself may be a transient and even reversible phenomenon, reflecting a dynamic balance of regulators for and against MPT.[118] Independent of MPT, families of proteins located in the mitochondrial outer membrane, Bax and Bcl-X, exert countervailing influences on cytochrome c release, favoring and inhibiting release, respectively.[110] Some proteins important in the apoptotic cascade, such as APAF-1, may be located in the intermembrane space.[119] The significance and roles of the proteins, and the impact of their submitochondrial localization, remain incompletely understood. A predisposition to myocyte apoptosis might contribute to the loss of myocytes observed in congestive heart failure[120] and in the aging heart.[121]

Mitochondrial calcium accumulation

Cytosolic calcium content increases during myocardial ischemia and reperfusion[122] leading to calcium accumulation by mitochondria via uptake by the inner membrane calcium uniporter.[123,124] Increased matrix calcium content leads to mitochondrial calcium release and reuptake, generating energy-consuming futile cycles that divert use of the inner membrane proton gradient to cation transport instead of to energy production.[124] Mitochondrial calcium loading predisposes to MPT.[113,114] SSM have a decreased capacity for calcium accumulation (Fig. 9).[90] Furthermore, calcium loading in SSM led to the release of cytochrome c whereas calcium loading in IFM did not result in cytochrome c release, even when the capacity of IFM to retain calcium was exceeded.[90] Elevated calcium content in the matrix activates proteases and phospholipase A_2, degrading proteins and phospholipids.[50] Matrix dense bodies visible by electron microscopy are a hallmark of irreversible mitochondrial damage secondary to calcium accumulation.

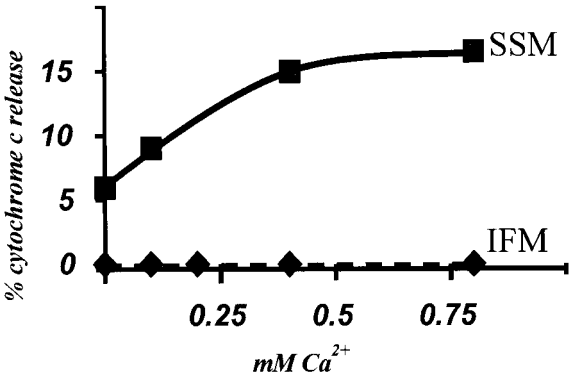

Figure 9 Subsarcolemmal mitochondria (SSM) have a decreased capacity to accumulate calcium compared to IFM. SSM release cytochrome c in response to calcium accumulation while IFM do not (the data are redrawn from *American Journal of Physiology* 1986; **250**: H741–H748).

Mitochondria as Sources and Targets of Injury in Specific Cardiac Disease States

Ischemia

Ischemia progressively damages mitochondria

Mitochondrial ultrastructural[125] and functional[126] injury occur early in the course of ischemia, and progresses during ischemia.[87,126] Ischemic damage to mitochondrial respiration also progresses as the duration of ischemia increases (Fig. 10).[87,93,99] Ten to 20 min of ischemia decreases complex I activity.[99,126] Damage to the phosphorylation apparatus, including complex V[99] and the adenine nucleotide transporter,[33,127] also occurs early in ischemia. However, mitochondrial oxidative function and cardiac contractile function recover following these periods of ischemia.[126] As ischemia continues for 30 and 45 min, at the onset of irreversible myocyte damage a second defect occurs in the ETC distal to complex

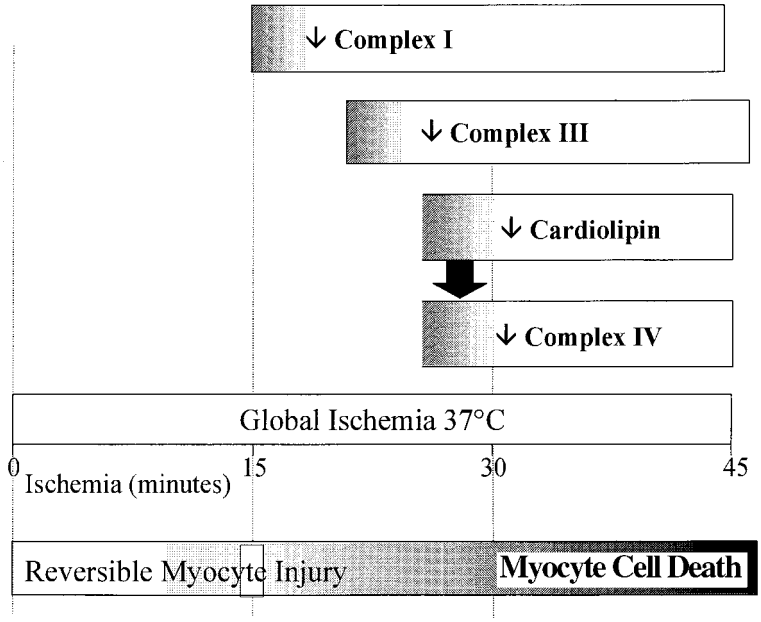

Figure 10 The progression of ischemic damage to SSM in the isolated perfused heart is shown. Fifteen minutes of ischemia leads to a complex I defect that is reversible with reperfusion. Complex III activity decreases by 25 min of ischemia as observed in the isolated perfused rat heart. By 30 min of ischemia, cardiolipin content decreases in SSM and oxidative phosphorylation through complex IV is decreased. Cardiolipin content and oxidative phosphorylation through complex IV remain decreased at 45 min ischemia. The onset of the transition from reversible to irreversible myocyte damage occurs at approximately 30 min of ischemia.

I.[87] Ischemic damage in the distal ETC involves both complexes III[110] and IV[87] (Fig. 6). Thus, while a complex I defect occurs early, as ischemia continues damage progresses to involve complexes III and IV.

ADP-stimulated respiration (state 3) is decreased during ischemia. Damage to the phosphorylation apparatus does not explain the decrease in state 3 respiration observed as ischemia progresses since dinitrophenol-uncoupled respiration is also decreased, localizing the functionally significant site of damage to the ETC.[87]

Complex V catalyzes ATP hydrolysis during ischemia, the reverse reaction of ADP phosphorylation that occurs during normal oxidative metabolism.[128] The enhanced consumption of ATP during ischemia accelerates the time course of ischemic injury. Cardiac myocytes contain a peptide inhibitor that binds to complex V during ischemia and blocks the hydrolysis of ATP.[129] Inhibition of complex V by ATPase inhibitor protein is cardioprotective during ischemia.[129]

Ischemic damage to the ETC is physiologically significant in terms of both loss of metabolic function and gain of pathologic alteration. The ischemia-induced decrease in the activity of complex IV is observed under both submaximally and maximally expressed rates of oxidative phosphorylation through complex IV.[87] Ischemic damage increases the control strength of the electron transport chain, including complex I and complex III.[85]

Molecular targets of ischemic damage to the ETC

Complex I is the initial site of ischemic damage to the ETC. Complex I activity decreases during ischemia secondary to a decrease in the NADH dehydrogenase component of the complex,[99,130] possibly due to the loss of the flavin mononucleotide coenzyme.[131] Damage to the NADH dehydrogenase component of complex I can increase electron leak and the production of reactive oxygen species.[39] Ischemic damage then progresses to involve complexes III and IV in the distal ETC.

Ischemia damages complex III by functional inactivation of the iron–sulfur protein (ISP) subunit in the isolated rat heart.[109] The ISP is a 22 kDa protein that contains a 2 Fe–2 S redox-active iron–sulfur cluster. Ischemic damage to the ISP results in loss of the EPR signal of the iron–sulfur cluster without loss of the ISP peptide, suggesting that ischemia results in disruption of the cluster without degradation of the ISP subunit.[109] Conserved cysteine and histidine residues are ligands for the Fe atoms in the cluster.[132] *In vitro* oxidative damage to the histidine residues leads to loss of complex III activity.[133] In addition to the ligands of the iron

atoms, the integrity of the cluster requires that the native conformation of ISP be preserved. An intramolecular disulfide bond between two highly conserved cysteine residues remote from the cluster itself is required to preserve the integrity of the cluster.[134] These sites provide likely targets for ischemia to disrupt the 2 Fe–2 S cluster without a loss of the peptide.

Complex IV is a membrane-associated enzyme complex composed of 13 peptide subunits, including catalytic, structural, and regulatory subunits.[31] Functional assays of complex IV systematically excluded ischemic damage to catalytic, regulatory, and structural subunits, including subunits that bind cytochrome c.[87] Ischemic damage to a protein subunit of complex IV is an unlikely mechanism for the ischemic defect in SSM in the rabbit heart.[87] Complex IV requires a lipid environment in the inner membrane enriched in cardiolipin[135,136] in addition to the integrity of peptide subunits for optimal activity. Ischemia decreases the content of cardiolipin in concert with the onset of the complex IV defect during the time course of ischemia in the isolated rabbit heart.[137] While cardiolipin content was decreased, the content of the remaining phospholipids was preserved.[137] The composition of the remaining cardiolipin was unaltered.[137] Mitochondrial cardiolipin content decreased in both *in vitro*[138,139] and *in vivo*[140,141] experimental models of myocardial ischemia.

The mitochondrial inner membrane is enriched in cardiolipin, a phospholipid unique to mitochondria.[142] Cardiolipin is a diphosphatidylglycerol with two diacylglycerol residues.[142] Cardiolipin per se is highly enriched in oxidatively-sensitive acyl-groups, containing 90–95% linoleic acid (C18:2).[137,143] The two double bonds in C18:2 can rearrange to a resonance-stabilized, conjugated diene following free radical-mediated abstraction of an allylic hydrogen.[144] C18:2 readily participates in free radical-initiated reactions in the membrane, with the formation of lipid peroxides. While oxidatively altered acyl-groups were not detected in cardiolipin from mitochondria from ischemic hearts, lipid peroxy-groups are unstable and may not be available for detection. Decomposition of lipid peroxides in cardiolipin can lead to the direct destruction of cardiolipin[144] or to covalent phospholipid–protein complexes that render the phospholipid no longer detectable as a phospholipid.[146]

Ischemic damage to cardiolipin is selective because the contents of other mitochondrial phospholipids remain unaltered during ischemia in the isolated rabbit heart.[137] The decrease in cardiolipin content occurs in the setting of preserved integrity of the inner membrane measured both by functional and morphologic criteria. The inner membrane retains the capacity to support a proton gradient.[146] State respiration is not increased.[87] Morphologic evidence of increased disruption of *in situ* and isolated populations of mitochondria is not observed using electron microscopy.[87] Ischemia does increase the viscosity of the inner membrane,[139,147] possibly as a consequence of cardiolipin depletion.[139] Loss of membrane fluidity decreases the activity of inner membrane transport systems and potentially of ETC complexes as well.[148]

Oxidative damage to mitochondria occurs during ischemia

The ETC is a probable source of reactive oxygen species that damage cardiolipin,[39–41] even during ischemia. Inhibitor blockade of the ETC decreases oxyradical production in isolated cardiac myocytes during simulated ischemia.[47] During ischemia, free radical generation occurs in myocardial tissue.[46,47] In the isolated rabbit heart, blockade of the proximal ETC with rotenone prevents the ischemia-induced decrease in cardiolipin content in SSM, consistent with the notion of oxidative damage to cardiolipin mediated by the ETC (Lesnefsky and Hoppel, unpublished data).

Ischemia also leads to oxidative damage to mitochondrial proteins. Ischemia decreases mitochondrial protein sulfhydryl (SH) content[148,149] and increases oxidatively-modified proteins detected by the carbonyl assay.[150] The mitochondrial ratio of reduced:oxidized glutathione decreases during ischemia, confirming a shift in redox state toward increased SH-oxidation.[151] Oxidative protein modifications accelerate degradation by proteases, which are activated by ischemia.[153] Thus, during ischemia there is both functional and chemical evidence of oxidative damage.

Ischemia decreases mitochondrial antioxidant defense, further enhancing the likelihood of oxidative damage. MnSOD activity decreases while cytosolic SOD activity remains unchanged.[152] Ischemia decreased mitochondrial glutathione peroxidase activity, a major source of the detoxification of H_2O_2.[152]

Ischemia and release of cytochrome c

Ischemia predisposes mitochondria to release cytochrome c from the intermembrane space.[153] Cytochrome c is detected in the cytosol at 30 min of ischemia in the rabbit heart.[153] The content of cytochrome c decreases in SSM at 30 min of ischemia,

concomitant with cardiolipin loss in SSM.[87,137] The production of oxidants, de-energization secondary to cessation of electron transport, calcium accumulation, and sulfhydryl oxidation all increase the likelihood of MPT, with subsequent release of cytochrome c. Loss of cardiolipin during ischemia may also cause loss of cytochrome c from the inner membrane, delocalizing cytochrome c from the inner membrane space. Cytochrome c is highly positively charged at physiologic pH (pI 10.3) due to the presence of multiple lysine residues.[154] Cardiolipin contains negatively-charged phosphatidic acid head groups (pK_a 4.8)[142] that bind cytochrome c.[155,156] Ischemia-induced loss of cardiolipin thus may predispose to cytochrome c release.

Mitochondrial calcium overload contributes to myocyte damage

Ischemia leads to mitochondrial calcium loading.[122] Since mitochondrial calcium uptake during ischemia occurs via the uniporter,[123] blockade of the uniporter with ruthenium red can determine if mitochondrial calcium uptake during ischemia leads to cardiac injury. Treatment of isolated hearts with ruthenium red reduced mitochondrial calcium uptake and ischemic myocardial injury.[123] If calcium-mediated injury is based upon the activation of PLA_2, a generalized decrease in mitochondrial phospholipid content with a corresponding increase in lysophospholipids would be expected.[50] This finding was not observed during ischemia.[137] Total mitochondrial phospholipid content did not decrease in SSM following 45 min of ischemia in the rabbit heart,[137] 60 min of global ischemia in the rat heart,[138] nor 120 min of regional ischemia in the pig heart.[141] Thus, generalized PLA_2 activation appears to be an unlikely mechanism for calcium-mediated mitochondrial damage during cardiac ischemia.

Ischemic damage is greater in SSM than in IFM

SSM sustain a more rapid onset of ischemic damage than do IFM in multiple experimental models.[33,87,88,93,140,147] An increased susceptibility of SSM to ischemic damage has been observed in isolated rat and rabbit hearts *in vitro*[33,87] and during regional ischemia *in vivo.*[140] In the isolated, perfused rabbit heart, at 30 and 45 min of ischemia, SSM exhibit decreased oxidative phosphorylation through complex IV and the distal ETC[87] and loss of cardiolipin[137] and cytochrome c[87] content. In contrast, IFM only display decreases in the oxidation of glutamate, the initial step in the progression of ischemic mitochondrial damage.[87] SSM produce reactive oxygen species following ischemic damage, while oxyradicals production by IFM is not enhanced.[147] The increased damage may occur secondarily either to their location in the subsarcolemmal compartment of the myocyte, or due to a greater intrinsic susceptibility to damage during ischemia. Even in the baseline state, SSM display an increased sensitivity to calcium-mediated damage compared to IFM,[90] raising the possibility that SSM inherently are more sensitive to ischemic damage than are IFM. The selectivity of ischemic damage to SSM provides a mechanism whereby damage to a subset of mitochondria leads to myocyte death.

Reperfusion

Reperfusion damage to the ETC

Recovery of mitochondrial function during reperfusion depends in part on the duration of the preceding ischemia. Following brief periods of ischemia, decreased rates of glutamate oxidation improve during reperfusion.[126] In contrast, following the onset of ischemic damage to complexes III and IV, the rate of oxidative phosphorylation further deteriorates during reperfusion,[157] suggesting that additional mitochondrial injury occurs.

The activities of ETC complexes I[126] and III[157] decrease during reperfusion. The sites of damage to these complexes during reperfusion is unknown. Although complex IV activity is decreased by ischemia and reperfusion,[139] damage during ischemia dominates, since the rate of oxidative phosphorylation through complex IV in intact mitochondria does not further deteriorate during reperfusion (Lesnefsky and Hoppel, unpublished data). Consistent with cardiolipin loss as the mechanism of the complex IV defect in SSM, cardiolipin content in SSM does not further deteriorate during early reperfusion in the isolated heart *in vitro* (Lesnefsky and Hoppel, unpublished data) or during reperfusion following regional ischemia *in vivo.*[140]

Mitochondria mediate oxidative damage during reperfusion

Ischemic damage to the ETC leads to increased $\bullet O_2^-$ and H_2O_2 generation during reperfusion.[105,158–161] The oxidation of either glutamate or succinate increases $\bullet O_2^-$ generation during reperfusion. During reperfusion, mitochondria generate reactive

oxygen species[105,146,159,160] that lead to additional mitochondrial[105,158,161] and myocyte injury.[158,159,161] Post-ischemic mitochondria generate the reactive and damaging hydroxyl radical (●OH).[105,160] In the isolated perfused heart, reperfusion following 30 min of ischemia increases oxyradical generation measured by ESR spin-trapping technique.[158] Increased mitochondrial ●OH generation also has been detected by the salicylate hydroxylation technique.[160] A critical amount of damage to the ETC by ischemia is required for the generation of reactive oxygen species. Normal cardiac mitochondria generate little ●OH, while intermediate degrees of damage markedly accentuate ●OH production. However, severe injury resulting in markedly reduced oxygen consumption greatly decreases ●OH formation.[105]

Reperfusion-induced declines in cardiac function and accumulation of oxidatively damaged lipids are diminished when mitochondrial respiration was reversibly inhibited during early reperfusion using amytal,[158] underlining the physiologic significance of mitochondrial oxyradical production. In contrast, inhibition of complex IV with CN^- does not confer protection.[158] Amytal treatment helps to dissect the relative contributions of complexes I and III in the genesis of oxidative damage.[162] Amytal blocks complex I near the binding site for ubiquinol, the electron acceptor from complex I.[162] Block of complex I at this distal site in electron flow through the complex enhances reduction of the NADH dehydrogenase in complex I, increasing electron leak from complex I to form reactive oxygen species.[39] Thus, rotenone blockade should enhance, not decrease, oxyradical production by complex I. Therefore, the observed decrease in the production of oxyradicals and improved myocardial protection in the presence of amytal strongly suggest that the site of production of reactive oxygen species occurs distal to the site of amytal block. Myxothiazole blocks electron entry into complex III[163] and blunts the production of reactive oxygen species by isolated mitochondria[164] and by cardiomyocytes.[47] Thus, blockage of electron influx into complex III is a likely mechanism of amytal-mediated protection.

Ischemic damage to complex IV also can contribute to oxidative damage during reperfusion. While direct production of reactive oxygen species by complex IV does not occur, inhibition of complex IV enhances oxidant-mediated cell death *in vitro*,[165] and does not protect against myocardial oxidative damage during reperfusion.[158] Complex IV modulates the redox status of upstream ETC complexes (Fig. 6). Electron flow through complex IV is under respiratory control or independent of respiratory control ("uncoupled").[35] Uncoupled electron flow through complex IV decreases the relative reduction of complexes I and III,[35,36] decreasing the likelihood of electron leak by these complexes to form oxyradicals. Ischemia-induced decreases in complex IV activity predispose to oxidant production by removing an antioxidant mechanism in the ETC.

Dysregulation of substrate oxidation during reperfusion

Damage due to ischemia or reperfusion disrupts the tight regulation of substrate selection for mitochondrial oxidation, having a negative impact on cardiac metabolic and mechanical function during reperfusion.[75,76] Cardiac recovery is enhanced by the oxidation of carbohydrates, rather than of fatty acids, during early reperfusion.[75,76] Reperfusion leads to an increase in uncoupled respiration that is observed in isolated mitochondria.[75–77] The increase in uncoupled respiration is manifested as an excess oxidation of substrates compared to energy production during reperfusion, decreasing the metabolic efficiency of the reperfused heart. The uncoupling of substrate oxidation from energy demand is enhanced with lipid substrates compared to carbohydrate substrates.[166] Reperfusion leads to the loss of regulation of fatty acid oxidation by CPT-I, probably secondarily to a decrease in content of malonyl-CoA.[168] The content of malonyl-CoA decreases during ischemia and further decreases during reperfusion.[168] As a consequence of increased supply of non-esterified fatty acids present in the intact organism during ischemia, the control strength at CPT-I is further attenuated due to the increased cytosolic content of long-chain acyl-CoAs. Whether the acyl-CoA modulation of CPT-I activity dominates or modifies intrinsic changes in the malonyl-CoA sensitivity of CPT-I remains to be elucidated.

Reperfusion predisposes to onset of mitochondrial permeability transition (MPT)

Mitochondrial oxidative damage is enhanced during reperfusion.[161] Increased calcium enhances mitochondrial calcium loading.[122–124] Enhanced mitochondrial oxidative damage and calcium loading predispose to the onset of MPT, which has been observed in reperfused myocardium *in situ* during early reperfusion.[112,113] Based upon their greater sensitivity to calcium-mediated damage,[90] MPT formation during reperfusion may involve largely

SSM. Inhibitors of MPT decrease myocardial damage during ischemia and reperfusion.[169] MPT appears to be reversible during reperfusion, with closure of the permeability transition pore enhanced by pyruvate oxidation during early reperfusion.[170] The contributions of MPT as an effector of oxidative and calcium-mediated processes to reperfusion damage requires further investigation.

Ischemic preconditioning

Brief periods of ischemia decrease the myocardial damage that results from a subsequent prolonged period of ischemia.[125] Ischemic preconditioning ameliorates ischemic mitochondrial damage, improving rates of oxidative phosphorylation toward rates observed in mitochondria from non-ischemic hearts.[171] Preconditioning protection is mediated in part via activation of adenosine (A-1) receptors on the plasma membrane.[172] A-1 receptor activation leads to activation of protein kinase C, including the ε isoform.[172] A major downstream effector of preconditioning protection is activation of the ATP-dependent potassium channel (K-ATP_{mito}) in the inner mitochondrial membrane.[172,173] Activation of K-ATP_{mito} channels leads to partial depolarization of the inner membrane[173] and to partial uncoupling with increased oxygen consumption and decreased ATP synthesis.[171] Mitochondrial calcium release also is increased.[171] Perhaps as a consequence of the increase in respiration, the oxidation of $FADH_2$ is increased.[173] The partial uncoupling of respiration may decrease the production of oxidants by mitochondria. Calcium release and less oxidative damage would be expected to decrease the likelihood of MPT and cytochrome c release. The mechanisms of mitochondrial-driven preconditioning-mediated protection will require further study. The mechanism by which protein kinase C activates the K-ATP_{mito} channel remains unclear at present.

Aging

Aging decreases oxidative phosphorylation selectively in IFM

Mitochondrial oxidative metabolism declines with age in many tissues. Oxidative phosphorylation was studied in SSM and IFM from 6-month adult and 24-month aging hearts using the Fischer 344 rat heart model of aging.[93,96] Aging decreases the protein yield and rate of oxidative phosphorylation in IFM.[91,96] In contrast to aging defects in IFM, the protein yield of SSM and the rate of oxidative phosphorylation in SSM remains unaltered by aging. Aging decreases the activity of ETC complexes III[96] and IV[91] in IFM. The activity of complexes I and II does not decrease with age in IFM.[91]

Aging-related decreases in fatty acid oxidation[138,174] and complex IV activity[148,176] previously described in mixed populations of cardiac mitochondria were localized to IFM.[92] In previous work by other laboratories, heart mitochondria isolated by tissue homogenization alone[177] that were expected to yield a population consisting of SSM, did not exhibit aging-related decreases in mitochondrial respiration. Previous results have been reconciled by the finding that aging-related decrements in mitochondrial respiration occur selectively in IFM.

An appreciation of the selective effect of aging on IFM is critical to the study of aging-related alterations in the heart. The use of tissue homogenization alone to isolate cardiac mitochondria paradoxically targets for study the SSM population, which is without aging-related oxidative defects. Recent studies of mitochondrial physiology in the aging heart, including the production of reactive oxygen species[178] and protein import,[179] found that aging did not alter these processes. However, the techniques of mitochondrial isolation used in these studies very likely yielded only SSM. The study of IFM, in which key aging-related alterations in oxidative phosphorylation reside, without the confounding effects of admixed SSM that remain unaltered by aging, will facilitate the study of mitochondria-related mechanisms of dysfunction in the aging heart.

Molecular defects in complexes III and IV in aging IFM

Aging decreased the rate of oxidative phosphorylation stimulated by TMPD-ascorbate, an electron donor to complex IV via cytochrome c (Fig. 6) in IFM.[92] The rate of uncoupled respiration was decreased, localizing the defect to the ETC.[92] The defect in electron transport was present under both submaximally and maximally expressed rates of oxidative phosphorylation.[92] Complex IV activity measured in permeabilized mitochondria in the presence of exogenous cytochrome c remained decreased in IFM, localizing the aging defect to complex IV rather than to cytochrome c. The decrease in complex IV activity observed in permeabilized IFM from

aging hearts was reversed by the addition of exogenous phospholipid liposomes.[92] In a mixed population of cardiac mitochondria, aging-related decreases in complex IV activity were reversed by the addition of the phospholipid cardiolipin.[176] These findings strongly suggest that the aging decrease in complex IV is secondary to a defect in the phospholipid environment, probably cardiolipin, of complex IV.

Complex IV requires cardiolipin,[142] especially cardiolipin enriched in unsaturated acyl-groups,[143] for optimal activity. The findings in aging IFM suggest that an aging-induced cardiolipin defect participates in the genesis of the complex IV defect. However, the content of cardiolipin, quantified by lipid phosphorous,[143] is preserved in IFM from aging hearts (Moghaddas, Lesnefsky, Hoppel, unpublished data). The recovery of cardiolipin from mitochondrial phospholipids was excellent. A previous study only estimated cardiolipin content by UV absorbance (a function of acyl-group composition) relative to the other phospholipids without independent quantitation, suggesting that a decrease in relative cardiolipin composition occurred in aging heart mitochondria.[148,176] The possible role of aging-related alterations in the composition, rather than in the content of cardiolipin, in IFM requires further study.

The rate of oxidative phosphorylation stimulated by durohydroquinone, an electron donor to complex III, was decreased by aging in IFM, but not in SSM.[97] The rate of uncoupled respiration was decreased, localizing the aging defect to the electron transport chain. Complex III activity was decreased by aging in IFM, but remained unchanged in SSM.[97] The decrease in complex III activity was observed under both maximal and submaximal conditions.[97] Studies were performed to localize the site of the aging defect in complex III. Aging did not alter the content of catalytic centers of complex III (cytochromes b and c_1 and iron–sulfur protein). Functional studies of complex III activity localized the aging defect to the cytochrome c binding site of complex III.[97] However, the subunit peptides (subunits VIII, X, and cytochrome c_1) that compose the cytochrome c binding site were present in complex III isolated from aging IFM.[97] Thus, modification, rather than loss, of peptide subunits that constitute the cytochrome c binding site is a likely mechanism of the aging defect in complex III.[97]

Aging increases oxidative damage in cardiac mitochondria

Aging defects present in cardiac mitochondria are probable sources of the increase in chronic oxidative injury in aging tissues.[180,181] Mitochondria isolated from senescent animals exhibit higher rates of oxyradical production than do those from young animals. H_2O_2 generation increases by 25% in mitochondria from 24-month-old rats.[182] Cardiac mitochondria from elderly hearts sustain greater lipid peroxidation and injury following exposure to exogenous iron and H_2O_2.[183] Mitochondria from aging tissues have increased oxidative stress even in the baseline state.[51,63] Cardiac mitochondria isolated from elderly rats had an increased content of 8-hydroxydeoxyguanosine, a marker of oxidative mtDNA damage, compared to adult controls.[63] The mtDNA changes are observed in heart, but not in liver,[63] suggestive of an increased susceptibility to oxidative injury in cardiac mitochondria from elderly animals.

Aging-related mitochondrial defects may predispose to increased injury in the aging heart

The elderly heart sustains greater injury during ischemia and reperfusion compared to the adult heart in both patients[184] and experimental models.[185–188] There is greater oxidative damage,[186] including oxidative protein modification,[186] as well as calcium-mediated damage[122] compared to the adult heart. The enhanced calcium-mediated and oxidative damage in the aging heart suggests mitochondria-driven mechanisms of injury in the aging heart. The addition of ischemic mitochondrial damage to pre-existing aging-related mitochondrial defects is a likely mechanism of enhanced mitochondrial-derived injury in the aging heart.[109]

Complex III, implicated as a source of reactive oxygen species during reperfusion in the adult heart, is a probable source of enhanced oxidative damage during reperfusion in the aging heart.[109] The addition of ischemia-induced defects in complex III to the preexisting aging-related defects can enhance mitochondrial-derived injury during reperfusion. Ischemia damages the iron–sulfur subunit of complex III.[109] Aging causes a defect at the cytochrome c binding site.[97] Thus, at the onset of reperfusion in the aging heart, complex III in IFM contains sequential defects in the path of electron flow. The two partial blocks in sequence are likely to act in concert to further slow electron flow within complex III, increase the reduction of cytochrome b, and enhance production of reactive oxygen species compared to either defect alone. An increase in intracellular oxidative injury leads to cell death by necrosis or apoptosis, processes that are increased in the aging heart during reperfusion.

Cardiomyopathy and congestive heart failure

Considerable attention has been given to the potential role of mitochondrial defects in the genesis of dilated cardiomyopathy in the adult. As a model, inherited diseases of mitochondrial metabolism impair oxidative phosphorylation in the heart, leading to cardiac lactate production and globally hypocontractile, dilated ventricles. The phenotype of neonatal heart failure has been observed with defects in ETC complexes I[189] and IV.[190] Defects in mitochondrial fatty acid oxidation also lead to this phenotype.[191] While mutations in mtDNA impair ETC activity, as observed in complexes I and IV, defects in nuclear-encoded mitochondrial proteins also are frequent sources of cardiomyopathy.[97,192] Mutations in the nuclear genome not only account for defects referable to non-ETC proteins, but also delete or alter nuclear-encoded ETC subunit proteins, impairing the function of ETC complexes. The MnSOD-homozygote mouse develops a cardiomyopathy in the neonatal period, indicative of a mitochondrial-driven cardiac phenotype.[193] The neonatal cardiomyopathy phenotype provides insights into the mitochondrial mechanisms of cardiomyopathies observed in adults.

Experimental models support a role of mitochondrial defects in the genesis of cardiomyopathy. Syrian hamster cardiomyopathy provides an animal model of human dilated cardiomyopathy.[91] The cardiomyopathic hamster heart contains a defect selective to IFM, whereas SSM are unaffected.[91] IFM do not contain a defect in the ETC, but rather one localized to the inner membrane and phosphorylation apparatus,[91] expected to impair energy production. Decreases in mitochondrial respiration in experimental heart failure also are observed at the myocyte level using saponin-skinned muscle bundles obtained from dogs with pacing-induced dilated cardiomyopathy and heart failure.[194] These experimental approaches provide key data supporting decrease in mitochondrial respiration in cardiomyopathy and heart failure, data lacking in human hearts.

Enzyme activity of ETC complexes is decreased in hearts explanted from patients with end-stage heart failure.[195–200] Decreases in complexes I, III, and IV have been reported. Other studies have detected deletion mutations in mtDNA.[198,200] Unfortunately, data linking these two observations are, in general, lacking. One report attempted to link the two observations in a patient with cardiomyopathy who had a point mutation in the mtDNA cytochrome b gene and a concomitant decrease in complex III enzyme activity.[200] However, other studies have detected only neutral polymorphisms in the cytochrome b gene despite decreases in complex III activity.[197] Thus, the relationship of mtDNA mutations in cardiomyopathy to decreased activity of ETC complexes and decreased mitochondrial oxidative phosphorylation remains to be established.

The potential for mitochondrial-derived myocyte injury exists in the cardiomyopathic heart. In pacing-induced canine models of heart failure, mitochondrial production of reactive oxygen species is increased, with complex I the likely source.[195] In addition to an increased production of reactive oxygen species, mitochondria from the failing heart release cytochrome c. Tumor necrosis factor production is increased in failing hearts.[201,202] Tumor necrosis factor leads to the production of the second messenger ceramide,[203] which inhibits complex III.[204] Decreased activity of complex III has been observed in the failing heart.[203–205] Ceramide-mediated inhibition of complex III increases the production of reactive oxygen species[205] and predisposes to mitochondrial-driven apoptotic pathways.[205,206] Extra-mitochondrial localization of cytochrome c, accompanied by activation of the downstream effector, caspase 3, are present in failing human hearts.[206,207] Thus, via either the independent or concerted action of oxidative damage and cytochrome c release, mitochondria are likely contributors to the enhanced myocyte dropout observed in the congestive heart failure state.

Summary

Mitochondrial damage leads to loss of mitochondrial function, impairing energy production and cell physiology, and to the enhancement of pathologic function, producing oxidative-, calcium-, and apoptosis-mediated myocyte injury. Mitochondria are key intermediates of myocyte damage during ischemia and reperfusion. These organelles are likely contributors to the chronic, relentless myocyte injury that culminates in myocyte dropout in aging and heart failure. Studies of mitochondrial function in cardiac disease states using models ranging from isolated molecular constituents, through isolated mitochondria and intact cells, to tissues and isolated organs will continue to provide new insights into the mechanisms of mitochondrial damage and of mitochondrial-mediated myocyte injury.

Acknowledgements

This work was supported by Grants 1RO1AG12447, 1K04AG00676 and 1POAG

15885 from the National Institutes of Health, and by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs.

References

1. Cortese JD, Voglino AL, Hackenbrock CR. The ionic strength of the intermembrane space of intact mitochondria is not affected by the pH or volume of the intermembrane space. *Biochim Biophys Acta* 1992; **1100**: 189–197.
2. Gupte SS, Hackenbrock CR. Multidimensional diffusion modes and collision frequencies of cytochrome c with its redox partners. *J Biol Chem* 1988; **263**: 5241–5247.
3. Van der Klei IJ, Veenhuis M, Neupert W. A morphological view on mitochondrial protein targeting. *Microsc Res Tech* 1994; **27**: 284–293.
4. Brdiczka D, Walliman T. The importance of the outer mitochondrial compartment in regulation of energy metabolism. *Mol Cell Biochem* 1994; **133/134**: 69–83.
5. Hoppel CL, Kerner J, Turkaly P, Turkaly J,Tandler B. The malonyl-CoA-sensitive form of carnitine palmitoyltransferase is not localized exclusively in the outer membrane of rat liver mitochondria. *J Biol Chem* 1998; **273**: 23495–23503.
6. Fraser F, Zammit VA. Enrichment of carnitine palmitoyltransferase I and II in the contact sites of rat liver mitochondria. *Biochem J* 1998; **329**: 225–229.
7. Nicolay K, Rojo M, Wallimann T, Demel R, Hovius R. The role of contact sites between inner and outer mitochondrial membrane in energy transfer. *Biochim Biophys Acta* 1990; **1018**: 229–233.
8. Turkaly P, Kener J, Hoppel CL. A 22 kDa polyanion inhibits carnitine-dependent fatty acid oxidation in rat liver mitochondria. *FEBS Lett* 1999; **460**: 241–245.
9. Zammit VA, Fraser F, Orstorphine CG. Regulation of mitochondrial outer-membrane carnitine palmitoyltransferase (CPTI): role of membrane-topology. *Adv Enzyme Reg* 1997; **37**: 295–317.
10. Kerner J, Zaluzec E, Gage D, Bieber LL. Characterization of the malonyl-CoA-sensitive carnitine palmitoyltransferase (CPT_o) of a rat heart mitochondrial particle: Evidence that the catalytic unit is CPT_i. *J Biol Chem* 1994; **269**: 8209–8219.
11. Brdiczka D, Bucheler K, Kottke M, Adams V, Nalam VK. Characterization and metabolic function of mitochondrial contact sites. *Biochim Biophys Acta* 1990; **1018**: 234–238.
12. Kerner J, Hoppel CL. Fatty acid import into mitochondria. *Biochim Biophys Acta* 2000; **1486**: 1–17.
13. Opie LH. Cardiac metabolism—emergence, decline, and resurgence. Part II. *Cardiovasc Res* 1992; **26**: 817–830.
14. Goodwin GW, Ahmad F, Doenst T, Taegtmeyer H. Energy provision from glycogen, glucose, and fatty acids on adrenergic stimulation of isolated working rat hearts. *Am J Physiol* 1998; **274**: H1239–H1247.
15. Goodwin GW, Taegtmeyer H. Improved energy homeostasis of the heart in the metabolic state of exercise. *Am J Physiol* 2000; **279**: H1490–H1501.
16. Lopaschuck G. Regulation of carbohydrate metabolism in ischemia and reperfusion. *Am Heart J* 2000; **139**: S115–S119.
17. Behal RH, Buxton DB, Robertson JG, Olson MS. Regulation of pyruvate dehydrogenase multienzyme complex. *Annu Rev Nutr* 1993; **13**: 497–520.
18. Bowker-Kinley MM, Davis WI, Wu P, Harris RA, Popov KM. Evidence for existence of tissue-specific regulation of mammalian pyruvate dehydrogenase complex. *Biochem J* 1998; **329**: 191–196.
19. Wu P, Inskeep K, Bowker-Kinley MM, Popov KM, Harris RA. Mechanism responsible for inactivation of skeletal muscle pyruvate dehydrogenase complex in starvation and diabetes. *Diabetes* 1999; **48**: 1593–1599.
20. Gertz EW, Wisneski JA, Stanley WC, Neese RA. Myocardial substrate utilization during exercise in humans; Dual carbon-labeled carbohydrate isotope experiments. *J Clin Invest* 1988; **82**: 2017–2025.
21. McCormack JG, Halestrap AP, Denton RM. Role of calcium ions in regulation of mammalian intramitochondrial metabolism. *Physiol Rev* 1990; **70**: 391–425.
22. McGarry JD, Mills SE, Long CS, Foster DW. Observations on the affinity for carnitine, and malonyl-CoA sensitivity, of carnitine palmitoyltransferase I in animal and human tissues. *Biochem J* 1983; **214**: 21–28.
23. Saddik M, Gamble J, Witters LA, Lopaschuk GD. Acetyl-CoA carboxylase regulation of fatty acid oxidation in the heart. *J Biol Chem* 1993; **268**: 255836–25845.
24. Hamilton C, Saggerson ED. Malonyl-CoA metabolism in cardiac myocytes. *Biochem J* 2000; **350**: 61–67.
25. Velasco G, Geelen MJ, Del Pulgar TG, Guzman M. Malonyl-CoA-independent acute control of hepatic carnitine plamitoyltransferase I activity. Role of Ca^{2+}/calmodulin-dependent protein kinase II and cytoskeletal components. *J Biol Chem* 1998; **273**: 21497–21504.
26. Kudo N, Barr AJ, Barr RL, Desai S, Lopaschuk GD. High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5′-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. *J Biol Chem* 1995; **270**: 17513–17520.
27. Schagger H, Pfeiffer K. Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. *EMBO J* 2000; **19**: 1777–1783.
28. Sazanov LA, Peak-Chew SY, Fearnley IM, Walker JE. Resolution of the membrane domain of bovine complex I into subcomplexes: implications for the structural organization of the enzyme. *Biochemistry* 2000; **39**: 7229–7235.
29. Lancaster CR, Kroger A. Succinate: quinone oxidoreductases: new insights from X-ray crystal structures. *Biochim Biophys Acta* 2000; **1459**: 422–431.
30. Xia D, Yu CA, Kim H, Xia JZ, Kachurin AM, Zhang L, Yu L, Deisenhofer J. Crystal structure of the cytochrome bc1 complex from bovine heart mitochondria. *Science* 1997; **277**: 60–66.

31. Cooper CE, Nicholls P, Freedman JA. Cytochrome c oxidase: structure, function, and membrane topology of the polypeptide subunits. *Biochem Cell Biol* 1991; **69**: 586–607.
32. Mitchell P. Possible mechanisms of the protonmotive function of cytochrome systems. *J Theor Biol* 1976; **62**: 327–367.
33. Duan J, Karmazyn M. Relationship between oxidative phosphorylation and adenine nucleotide translocase activity of two populations of cardiac mitochondria and mechanical recovery of ischemic hearts following reperfusion. *Can J Physiol Pharmacol* 1989; **67**: 704–709.
34. Saraste M. Oxidative phosphorylation at the fin de siecle. *Science* 1999; **283**: 1488–1493.
35. Papa S, Guerrieri F, Capitanio N. A possible role of slips in cytochrome c oxidase in the antioxygen defense system of the cell. *Biosci Rep* 1997; **17**: 23–31.
36. Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation. III. The steady state. Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA, pp. 409–427, 1955.
37. Ricquier D, Bouillaud F. Mitochondrial uncoupling proteins: from mitochondria to the regulation of energy balance. *J Physiol* 2000; **529**: 3–10.
38. Boss O, Hagen T, Lowell BB. Uncoupling proteins 2 and 3: potential regulators of mitochondrial energy metabolism. *Diabetes* 2000; **49**: 143–156.
39. Turrens JF, Boveris A. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. *Biochem J* 1980; **191**: 421–427.
40. Turrens JF. Superoxide production by the mitochondrial respiratory chain. *Biosci Rep* 1997; **17**: 3–8.
41. Sugioka K, Nakano M, Totsune-Nakano H, Minakami H, Tero-Kubota S, Ikegami Y. Mechanism of O_2^- generation in reduction and oxidation cycle of ubiquinones in a model of mitochondrial electron transport systems. *Biochim Biophys Acta* 1988; **936**: 377–385.
42. Turrens JF, Alexandre A, Lehninger AL. Ubisemiquinone is the electron donor for superoxide formation by complex III of heart mitochondria. *Arch Biochem Biophys* 1985; **237**: 408–414.
43. Babcock GT, Varotsis C. Discrete steps in dioxygen activation—the cytochrome oxidase/O_2 reaction. *J Bioenerg Biomemb* 1993; **252**: 71–80.
44. Kwong LK, Sohal RS. Substrate and site specificity of hydrogen peroxide generation in mouse mitochondria. *Arch Biochem Biophys* 1998; **350**: 118–126.
45. Liu SS. Cooperation of a "reactive oxygen cycle" with the Q cycle and the proton cycle in the respiratory chain-superoxide generating and cycling mechanisms in mitochondria. *J Bioenerg Biomembr* 1999; **31**: 367–376.
46. Davies MJ. Direct detection of radical production in the ischaemic and reperfused myocardium: current status. *Free Rad Res Comm* 1989; **7**: 275–284.
47. Becker LB, Vanden Hoek TL, Shao Z-H, Li C-Q, Schumacker PT. Generation of superoxide in cardiomyocytes during ischemia before reperfusion. *Am J Physiol* 1999; **277**: H2240–H2246.
48. Demin OV, Kholodenko BN, Skulachev VP. A model of O_2^- generation in the complex III of the electron transport chain. *Mol Cell Biochem* 1998; **184**: 21–33.
49. Halliwell B, Gutteridge JMC. Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* 1984; **219**: 1–14.
50. Sevian A. Lipid Peroxidation, Membrane Damage, and Phospholipase A_2 action. *Cellular Antioxidant Defense Mechanisms*, Vol. II. CRC Press, Boca Raton, FL, 1988.
51. Richter C, Park JW, Ames BN. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc Nat Acad Sci* 1988; **85**: 6465–6467.
52. Radi R, Turrens JF, Chang LY, Bush KM, Crapo JD, Freeman BA. Detection of catalase in rat heart mitochondria. *J Biol Chem* 1991; **266**: 22028–22034.
53. Simmons TW, Jamall IS. Relative importance of intracellular glutathione peroxidase and catalase *in vivo* for prevention of peroxidation to the heart. *Cardiovasc Res* 1989; **23**: 774–779.
54. Lesnefsky EJ, Dauber IM, Horwitz LD. Myocardial sulfhydryl pool alterations occur during reperfusion after brief and prolonged myocardial ischemia *in vivo*. *Circ Res* 1991; **68**: 605–613.
55. Lesnefsky EJ. Reduction of infarct size by cell-permeable oxygen metabolite scavengers. *Free Rad Biol Med* 1992; **12**: 429–446.
56. Arai M, Imai H, Koumura T, Yoshida M, Emoto K, Umeda M, Chiba N, Nakagawa Y. Mitochondrial phospholipid hydroperoxide glutathione peroxidase plays a major role in preventing oxidative injury to cells. *J Biol Chem* 1999; **274**: 4924–4933.
57. Raha S, McEachern GE, Myint AT, Robinson BH. Superoxides from mitochondrial complex III: the role of manganese superoxide dismutase. *Free Rad Biol Med* 2000; **29**: 170–180.
58. Gravina SA, Mieyal JJ. Thioltransferase is a specific glutathionyl mixed disulfide oxidoreductase. *Biochemistry* 1993; **32**: 3368–3376.
59. Starke DW, Chen Y, Bapna CP, Lesnefsky EJ, Mieyal JJ. Sensitivity of protein sulfhydryl repair enzymes to oxidative stress. *Free Rad Biol Med* 1997; **23**: 373–384.
60. Croteau DL, Stierum RH, Bohr VA. Mitochondrial DNA repair pathways. *Mutat Res* 1999; **434**: 137–148.
61. Bohr VA, Anson RM. Mitochondrial DNA repair pathways. *J Bioenerg Biomembr* 1999; **31**: 391–398.
62. Takasawa M, Hayakawa M, Sugiyama S, Hattori K, Ito T, Ozawa T. Age-associated damage in mitochondrial function in rat hearts. *Exp Gerontol* 1993; **28**: 269–280.
63. Wallace DC. Mitochondrial defects in cardiomyopathy and neuromuscular disease. *Am Heart J* 2000; **139**: S70–S85.
64. Corral-Debrinski M, Shoffner JM, Lott MT, Wallace DC. Association of mitochondrial DNA damage with aging and coronary atherosclerotic heart disease. *Mutat Res* 1992; **275**: 169–180.
65. Nagley P, Mackay IR, Baumer A, Maxwell RJ, Vaillant F, Wang ZX, Zhang C, Linnane AW. Mitochondrial DNA mutation associated with aging and degenerative disease. *Ann N Y Acad Sci* 1992; **673**: 92–102.
66. Lee CM, Lopez ME, Weindruch R, Aiken JM. Association of age-related mitochondrial ab-

normalities with skeletal muscle fiber atrophy. *Free Rad Biol Med* 1998; **25**: 964–972.
67. Taylor RW, Chinnery PF, Turnbull DM, Lightowlers RN. *In-vitro* genetic modification of mitochondrial function. *Hum Reprod* 2000; **15** Suppl 2: 79–85.
68. Muller-Hocker J. Cytochrome-c-oxidase deficient cardiomyocytes in the human heart—an age-related phenomenon. A histochemical ultracytochemical study. *Am J Pathol* 1989; **134**: 1167–1173.
69. Koehler CM. Protein translocation pathways of the mitochondrion. *FEBS Lett* 2000; **476**: 27–31.
70. Lithgow T. Targeting of proteins to mitochondria. *FEBS Lett* 2000; **476**: 22–26.
71. Hennig B, Koehler H, Neupert W. Receptor sites involved in posttranslational transport of apocytochrome c into mitochondria: specificity, affinity, and number of sites. *Proc Natl Acad Sci* 1983; **80**: 4963–4967.
72. Langer T, Neupert W. Regulated protein degradation in mitochondria. *Experientia* 1996; **52**: 1069–1076.
73. Ning XH, Zhang J, Liu J, Ye Y, Chen SH, From AH, Bache RJ, Portman MA. Signaling and expression for mitochondrial membrane proteins during left ventricular remodeling and contractile failure after myocardial infarction. *J Am Coll Cardiol* 2000; **36**: 282–287.
74. Neely JR, Rovetto MJ, Whitmer JT, Morgan HE. Effects of ischemia on function and metabolism of the isolated working rat heart. *Am J Physiol* 1973; **225**: 651–658.
75. Eberli FR, Weinberg EO, Grice WN, Horowitz GL, Apstein CS. Protective effect of increased glycolytic substrate against systolic and diastolic dysfunction and increased coronary resistance from prolonged global under perfusion and reperfusion in isolated rabbit hearts perfused with erythrocyte suspensions. *Circ Res* 1991; **68**: 466–481.
76. Lopaschuk GD, Spafford MA. Energy substrate utilization by isolated working hearts from newborn rabbits. *Am J Physiol* 1990; **258**: H1274–H1280.
77. Doenst T, Guthrie PH, Chemnitius JM, Zech R, Taegtmeyer H. Fasting, lactate, and insulin improve ischemia tolerance in rat heart: a comparison with ischemic preconditioning. *Am J Physiol* 1996; **270**: H1607–H1615.
78. Cairns CB, Ferroggiaro AA, Walther JM, Harken AH, Banerjee A. Postischemic administration of succinate reverses the impairment of oxidative phosphorylation after cardiac ischemia and reperfusion injury. *Circulation* 1997; **96** (Suppl II): II-260–II-265.
79. Di Lisa F, Blank PS, Colonna R, Gambassi G, Silverman HS, Stern MD, Hansford RG. Mitochondrial membrane potential in single living adult rat cardiac myocytes exposed to anoxia or metabolic inhibition. *J Physiol* 1995; **486**: 1–13.
80. Minezaki KK, Suleiman MS, Chapman RA. Changes in mitochondrial function induced in isolated guinea-pig ventricular myocytes by calcium overload. *J Physiol* 1994; **476**: 459–471.
81. Trollinger DR, Cascio WE, Lemasters JJ. Mitochondrial calcium transients in adult rabbit cardiac myocytes: inhibition by ruthenium red and artifacts caused by lysosomal loading of Ca(2+)-indicating fluorophores. *Biophys J* 2000; **79**: 39–50.
82. Swift LM, Sarvazyan N. Localization of dichlorofluorescin in cardiac myocytes: implications for assessment of oxidative stress. *Am J Physiol* 2000; **278**: H982–H990.
83. Groen AK, Wanders RJ, Westerhoff HV, Van Der Meer R, Tager JM. Quantification of the contribution of various steps to the control of mitochondrial respiration. *J Biol Chem* 1982; **257**: 2754–2757.
84. Gellerich FN, Bohnensack R, Kunz W. Control of mitochondrial respiration. The contribution of the adenine nucleotide translocator depends on the ATP- and ADP-consuming enzymes. *Biochim Biophys Acta* 1983; **722**: 381–391.
85. Borutaite V, Mildaziene V, Brown GC, Brand MD. Control and kinetic analysis of ischemia-damaged heart mitochondria: which parts of the oxidative phosphorylation system are affected by ischemia? *Biochim Biophys Acta* 1995; **1272**: 154–158.
86. Palmer JW, Tandler B, Hoppel CL. Biochemical properties of subsarcolemmal and interfibrillar mitochondria isolated from rat cardiac muscle. *J Biol Chem* 1977; **252**: 8731–8739.
87. Lesnefsky EJ, Tandler B, Ye J, Slabe TJ, Turkaly J, Hoppel CL. Myocardial ischemia decreases oxidative phosphorylation through complex IV in subsarcolemmal mitochondria. *Am J Physiol* 1997; **273**: H1544–H1554.
88. Shin G, Sugiyama M, Shoji T, Kagiyama A, Sato H, Ogura R. Detection of mitochondrial membrane damages in myocardial ischemia with ESR spin labeling technique. *J Mol Cell Cardiol* 1989; **21**: 1029–1036.
89. Palmer JW, Tandler B, Hoppel CL. Biochemical differences between subsarcolemmal and interfibrillar mitochondria from rat cardiac muscle: effects of procedural manipulations. *Archiv Biochem Biophys* 1985; **236**: 691–702.
90. Palmer JW, Tandler B, Hoppel CL. Heterogeneous response of subsarcolemmal heart mitochondria to calcium. *Am J Physiol* 1986; **250**: H741–H748.
91. Hoppel CL, Tandler B, Parland W, Turkaly JS, Albers LD. Hamster cardiomyopathy. A defect in oxidative phosphorylation in the cardiac interfibrillar mitochondria. *J Biol Chem* 1982; **257**: 1540–1548.
92. Fannin SW, Lesnefsky EJ, Slabe TJ, Hassan MO, Hoppel CL. Aging selectively decrease oxidative capacity in rat heart interfibrillar mitochondria. *Arch Biochem Biophys* 1999; **372**: 399–407.
93. Piper HM, Sezer O, Schleyer M, Schwartz P, Hutter JF, Spieckermann PG. Development of ischemia-induced damage in defined mitochondrial subpopulations. *J Mol Cell Cardiol* 1985; **17**: 885–896.
94. Saks VA, Kuznetsov AV, Kupriyanov VV, Miceli MV, Jacobus WE. Creatine kinase of rat heart mitochondria. The demonstration of functional coupling to oxidative phosphorylation in an inner membrane-matrix preparation. *J Biol Chem* 1985; **260**: 7757–7764.
95. Cortese JD, Voglino AL, Hackenbrock CR. Persistence of cytochrome c binding to membranes at physiological mitochondrial intermembrane space

ionic strength. *Biochim Biophys Acta* 1995; **1228**: 216–228.
96. Hoppel C, Cooper C. Studies on the nucleotide specificity of mitochondrial inner membrane particles. *Arch Biochem Biophys* 1969; **135**: 184–193.
97. Lesnefsky EJ, Gudz TI, Moghaddas S, Migita CT, Saito MI, Turkaly PJ, Hoppel CL. Aging decreases electron transport complex III activity in heart interfibrillar mitochondria by alteration of the cytochrome c binding site. *J Mol Cell Cardiol* 2001; **33**: 37–47.
98. Matsuno-Yagi A, Hatefi Y. Ubiquinol:cytochrome c oxidoreductase. Effects of inhibitors on reverse electron transfer from the iron-sulfur protein to cytochrome b. *J Biol Chem* 1999; **274**: 9283–9288.
99. Rouslin W. Mitochondrial complexes I, II, III, IV and V in myocardial ischemia and autolysis. *Am J Physiol* 1983; **244**: H743–H748.
100. Bittl JA, Weisfeldt ML, Jacobus WE. Creatine kinase of heart mitochondria. The progressive loss of enzyme activity during *in vivo* ischemia and its correlation to depressed myocardial function. *J Biol Chem* 1985; **260**: 208–214.
101. Corr PB, Snyder DW, Cain ME, Crafford WA Jr, Gross RW, Sobel BE. Electrophysiological effects of amphiphiles on canine purkinje fibers. Implications for dysrhythmia secondary to ischemia. *Circ Res* 1981; **49**: 354–363.
102. Corr PB, Sobel BE. Arrhythmogenic properties of phospholipid metabolites associated with myocardial ischemia. *Fed Proc* 1983; **42**: 2454–2459.
103. Corr PB, Gross RW, Sobel BE. Amphipathic metabolites and membrane dysfunction in ischemic myocardium. *Circ Res* 1984; **55**: 135–154.
104. Knabb MT, Saffitz JE, Corr PB, Sobel BE. The dependence of electrophysiological derangements on accumulation of endogenous long-chain acyl carnitine in hypoxic neonatal rat myocytes. *Circ Res* 1986; **58**: 230–240.
105. Otani H, Tanaka H, Inoue T, Umemoto M, Omoto K, Tanaka K, Sato T, Osako T, Masuda A, Nonoyama A, Kagawa T. *In vitro* study on contribution of oxidative metabolism of isolated rabbit heart mitochondria to myocardial reperfusion injury. *Circ Res* 1984; **55**: 168–175.
106. Lenaz G, Bovina C, Formiggini G, Castelli GP. Mitochondria, oxidative stress, and antioxidant defenses. *Acta Biochim Pol* 1999; **46**: 1–21.
107. Lenaz G, D'Aurelio M, Merlo Pich M, Genova ML, Ventura B, Bovina C, Formiggini G, Parenti Castelli G. Mitochondrial bioenergetics in aging. *Biochim Biophys Acta* 2000; **1459**: 397–404.
108. Turrens JF, Beconi M, Barilla J, Chavez UB, McCord JM. Mitochondrial generation of oxygen radicals during reoxygenation of ischemic tissues. *Free Rad Res Comm* 1991; **12–13**: 681–689.
109. Lesnefsky EJ, Gudz TI, Migita CT, Saito MI, Hassan MO, Turkaly PJ, Hoppel CL. Ischemic injury to mitochondrial electron transport in the aging heart: damage to the iron–sulfur protein subunit of electron transport complex III. *Arch Biochem Biophys* 2001; **385**: 117–128.
110. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; **281**: 1309–1316.
111. Yang J, Liu X, Bhalla K, Kim CN, Ibardo AM, Cai J, Peng TI, Jones DP, Wang X. Prevention of apoptosis by Bcl-2; release of cytochrome c from mitochondria blocked. *Science* 1997; **275**: 1129–1132.
112. Li F, Srinvasan A, Wang Y, Armstrong RC, Tomaselli KJ, Fritz L. Cells-specific induction of apoptosis by microinjection of cytochrome c, Bcl-x has activity independent of cytochrome c release. *J Biol Chem* 1997; **272**: 30299–30305.
113. Crompton M. The mitochondrial permeability transition pore and its role in cell death. *Biochem J* 1990; **341**: 233–249.
114. Kushnareva YE, Sokolove PM. Prooxidants open both the mitochondrial permeability transition pore and a low-conductance channel in the inner mitochondrial membrane. *Arch Biochem Biophys* 2000; **376**: 377–388.
115. Sobell S, Brdiczka D, Jahnke D, Schmidt A, Schlattner U, Wendt S, Wyss M, Walliman T. Octamerdimer transition of mitochondrial creatine kinase in heart disease. *J Mol Cell Cardiol* 1999; **31**: 857–866.
116. O'Gorman E, Beutner G, Dolder M, Koretsky AP, Brdiczka D, Walliman T. The role of creatine kinase in inhibition of mitochondrial permeability transition. *FEBS Lett* 1997; **414**: 253–257.
117. Stachowiak O, Dolder M, Walliman T, Richter C. Mitochondrial creatine kinase is a prime target of peroxynitrite-induced modification and inactivation. *J Biol Chem* 1998; **273**: 16694–16699.
118. Balakirev MYU, Khramtsov VV, Zimmer G. Modulation of the mitochondrial permeability transition by nitric oxide. *Eur J Biochem* 1997; **246**: 710–718.
119. Adachi S, Gottlieb RA, Babior BM. Lack of release of cytochrome C from mitochondria into cytosol early in the course of Fas-mediated apoptosis of Jurkat cells. *J Biol Chem* 1998; **273**: 19892–19894.
120. Petrovic D, Zorc-Pleskovic R, Zorc M. Apoptosis and proliferation of cardiomyocytes in heart failure of different etiologies. *Cardiovasc Pathol* 2000; **9**: 149–152.
121. Wie JY. Age and the cardiovascular system. *NEJM* 1992; **327**: 1735–1739.
122. Atake K, Chen D, Levitsky S, Jimenez E, Feinberg H. Effect of aging on intracellular Ca^{2+}, pHi, and contractility during ischemia and reperfusion. *Circulation* 1992; **86**: II371–II376.
123. Grover GJ, Dzwonczyk S, Sleph PG. Ruthenium red improves postischemic contractile function in isolated rat hearts. *J Cardiovasc Pharm* 1990; **16**: 783–789.
124. Gunter TE, Pfeiffer DR. Mechanisms by which mitochondria transport calcium. *Am J Physiol* 1990; **258**: C755–C786.
125. Murry CE, Richard VJ, Reimer KA, Jennings RB. Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circ Res* 1990; **66**: 913–931.
126. Flameng W, Andres J, Ferdinande P, Mattheussen M, Van Belle H. Mitochondrial function in myocardial stunning. *J Mol Cell Cardiol* 1991; **23**: 1–11.
127. Asimakis GK, Conti VR. Myocardial ischemia: correlation of mitochondrial adenine nucleotide and respiratory function. *J Mol Cell Cardiol* 1984; **16**: 439–448.
128. Rouslin W, Broge CW, Grupp IL. ATP depletion

and mitochondrial functional loss during ischemia in slow and fast heart-rate hearts. *Am P Physiol* 1990; **259**: H1759–H1766.
129. Rouslin W, Broge CW, Guerrieri F, Capozza G. ATPase activity, IF1 content, and proton conductivity of ESMP from control and ischemic slow and fast heart-rate hearts. *J Bioenerg Biomembr* 1995; **27**: 459–466.
130. Nohl H. Generation of superoxide radicals as byproduct of cellular respiration. *Ann Biol Clin* 1994; **52**: 199–204.
131. Rouslin W, Ranganathan S. Impaired function of mitochondrial electron transfer complex I in canine myocardial ischemia: loss of flavin mononucleotide. *J Mol Cell Cardiol* 1983; **15**: 537–542.
132. Kim H, Xia D, Yu CA, Xia JZ, Kachurin AM, Zhang L, Yu L, Deisenhofer J. Inhibitor binding changes domain mobility in the iron–sulfur protein of the mitochondrial bc1 complex from bovine heart. *Proc Natl Acad Sci* 1998; **95**: 8026–8033.
133. Miki T, Yu L, Yu CA. Hematoporphyrin-promoted photo inactivation of mitochondrial ubiquinol-cytochrome c reductase: selective destruction of the histidine ligands of the iron–sulfur cluster and protective effect of ubiquinone. *Biochemistry* 1991; **30**: 230–238.
134. Denke E, Merbitz-Zahradnik T, Hatzfeld OM, Snyder CH, Link TA, Trumpower BL. Alteration of the midpoint potential and catalytic activity of the rieske iron–sulfur protein by changes of amino acids forming hydrogen bonds to the iron–sulfur cluster. *J Biol Chem* 1998; **273**: 9085–9093.
135. Robinson NC, Strey F, Talbert L. Investigation of the essential boundary layer phospholipids of cytochrome c oxidase using triton X-100 delipidation. *Biochemistry* 1980; **19**: 3656–3661.
136. Vik SB, Capaldi RA. Lipid requirements for cytochrome c oxidase activity. *Biochemistry* 1997; **16**: 5755–5759.
137. Lesnefsky EJ, Slabe TJ, Stoll MSK, Minkler PJ, Hoppel CL. Myocardial ischemia selectively depletes cardiolipin in rabbit heart subsarcolemmal mitochondria. *Am J Physiol* 2001 (in press).
138. Edoute Y, van der Merwe E, Sanan D, Kotze JCN, Steinmann C, Lochner A. Normothermic ischemic cardiac arrest of the isolated working rat heart. *Circ Res* 1983; **53**: 663–678.
139. Paradies G, Petrosillo G, Pistolese M, Divenosa N, Serena D, Ruggiero FM. Lipid peroxidation and alterations to oxidative metabolism in mitochondria isolated from rat heart subjected to ischemia and reperfusion. *Free Rad Biol Med* 1999; **27**: 42–50.
140. Kayiyama K, Pauly DF, Hughes H, Yoon BY, Entman ML, McMillin-Wood JB. Protection by verapamil of mitochondrial glutathione equilibrium and phospholipid changes during reperfusion of ischemic canine myocardium. *Circ Res* 1987; **61**: 301–310.
141. Vasdev SC, Biro GP. Membrane changes induced by early myocardial ischemia in the dog. *Can J Biochem* 1980; **58**: 1112–1119.
142. Hoch FL. Cardiolipins and biomembrane function. *Biochim Biophys Acta* 1992; **1113**: 71–133.
143. Abramovitch DA, Marsh D, Powell GL. Activation of beef heart cytochrome c oxidase by cardiolipin and analogues of cardiolipin. *Biochim Biophys Acta* 1990; **1020**: 34–42.
144. O'Brien PJ. Intracellular mechanisms for the decomposition of a lipid peroxide. I. Decomposition of a lipid peroxide by metal ions, heme compounds, and nucleophiles. *Can J Biochem* 1969; **47**: 485–499.
145. Parinandi NL, Zwizinski CW, Schmid HHO. Free radical-induced alterations of myocardial membrane proteins. *Arch Biochem Biophys* 1991; **289**: 118–123.
146. Toleikis A, Dzeja P, Praskevicius A, Jasaitis A. Mitochondrial functions in ischemic myocardium. I. Proton electrochemical gradient, inner membrane permeability, calcium transport and oxidative phosphorylation in isolated mitochondria. *J Mol Cell Cardiol* 1979; **11**: 57–76.
147. Ueta H, Ogura R, Sugiyama M, Kagiyama A, Shin G. Spin trapping on cardiac submitochondrial particles isolated from ischemic and non-ischemic myocardium. *J Mol Cell Cardiol* 1990; **22**: 893–899.
148. Paradies G, Ruggiero FM, Petrosillo G, Quagliarello E. Age-dependent decrease in the cytochrome c oxidase activity and changes in phospholipids in rat-heart mitochondria. *Arch Gerontol Geriatr* 1993; **16**: 263–272.
149. Arduini A, Mezzetti A, Porreca E, Lapenna D, Dejulia J, Marzio L, Polidoro G, Cuccurullo F. Effect of ischemia and reperfusion on antioxidant enzymes and mitochondrial inner membrane proteins in perfused rat heart. *Biochim Biophys Acta* 1988; **970**: 113–121.
150. Pauly DF, Kirk KA, McMillin JB. Carnitine palmitoyltransferase in cardiac ischemia. A potential site for altered fatty acid metabolism. *Circ Res* 1991; **68**: 1085–1094.
151. Davies KJ, Delsignore ME. Protein damage and degradation by oxygen radicals. III. Modification of secondary and tertiary structure. *J Biol Chem* 1987; **262**: 9908–9913.
152. Shlafer M, Myers CL, Adkins S. Mitochondrial hydrogen peroxide generation and activities of glutathione peroxidase and superoxide dismutase following global ischemia. *J Mol Cell Cardiol* 1987; **19**: 1195–1206.
153. Fan T-HM, Xu J, Pain TS, Kreuz M, Cohen MV, Downey JM. Diazoxide inhibits mitochondrial cytochrome c release in ischemic rabbit myocardium. *Circulation* 1999; **100**: I–630 (abstract).
154. Flatmark T. Cytochrome c pI. *Acta Chem Scand* 1966; **20**: 1476–1486.
155. Shidoji Y, Hyaashi K, Komura S, Ohishi N, Yagi K. Loss of molecular interaction between cytochrome c and cardiolipin due to lipid peroxidation. *Biochem Biophys Res Comm* 1999; **264**: 343–347.
156. Rytomaa M, Kinnunen PKJ. Evidence for two distinct acidic phospholipid-binding sites in cytochrome c. *J Biol Chem* 1994; **269**: 1770–1774.
157. Veitch K, Hombreckx A, Caucheteux D, Pouleur H, Hue L. Global ischaemia induces a biphasic response of the mitochondrial respiratory chain. Anoxic pre-perfusion protects against ischaemic damage. *Biochem J* 1992; **281**: 709–715.
158. Ambrosio G, Zweier JL, Duilio C, Kuppusamy P, Santoro G, Elia PP, Tritto I, Crillo P, Condorelli

M, Chiariello M, Flaherty JT. Evidence that mitochondrial respiration is a source of potentially toxic oxygen free radical in intact rabbit hearts subjected to ischemia and reflow. *J Biol Chem* 1993; **268**: 18432–18541.
159. Sblafer M, Gallagher KP, Adkins S. Hydrogen peroxide generation by mitochondria isolated from regionally ischemic and nonischemic dog myocardium. *Bas Res Cardiol* 1990; **85**: 318–329.
160. Park JW, Chun YS, Kim YH, Kim CH, Kim MS. Ischemic preconditioning reduces Op6 generation and prevents respiratory impairment in the mitochondria of post-ischemic reperfused heart of rat. *Life Sci* 1997; **60**: 2207–2219.
161. Ambrosio G, Flaherty JT. Effects of the superoxide radical scavenger superoxide dismutase, and of the hyroxyl radical scavenger mannitol, on reperfusion injury in isolated rabbit hearts. *Cardiovasc Drugs Ther* 1992; **6**: 623–632.
162. Okun JG, Lummen P, Brandt U. Three classes of inhibitors share a common binding domain in mitochondrial complex I (NADH:ubiquinone oxidoreductase). *J Biol Chem* 1999; **274**: 2625–2630.
163. Trumpower BL. The protonmotive Q cycle. *J Biol Chem* 1990; **265**: 11409–11412.
164. Bandy B, Davison AJ. Mitochondrial mutations may increase oxidative stress: implications for carcinogenesis and aging. *Free Rad Biol Med* 1990; **8**: 523–539.
165. Dawson TL, Gores GL, Nieninen AL, Herman B, Lemasters JJ. Mitochondria as a source of reactive oxygen species during reductive stress in rat hepatocytes. *Am K Physiol* 1993; **264**: C961–C966.
166. Hermesh O, Kalderon B, Bar-Tana J. Mitochondria uncoupling by a long chain fatty acyl analogue. *J Biol Chem* 1998; **273**: 3937–3942.
167. Furman E, Sonn J, Acad BA, Dvir S, Kedem J. Relation between myocardial substrate utilization, oxygen consumption and regional oxygen balance in the dog heart *in vivo. Arch Int Physiol Biochim* 1986; **94**: 285–293.
168. Kennedy JA, Kiosoglous AJ, Murphy GA, Pelle MA, Horowitz JD. Effect of perhexiline and oxfenicine on myocardial function and metabolism during low-flow ischemia/reperfusion in the isolated rat heart. *J Cardiovasc Pharm* 2000; **36**: 794–801.
169. Griffiths EJ, Halestrap AP. Protection by cyclosporin A of ischemia/reperfusion-induced damage in isolated rat hearts. *J Mol Cell Cardiol* 1993; **25**: 1461–1469.
170. Crompton M. Mitochondrial intermembrane junctional complexes and their role in cell death. *J Physiol* 2000; **529**: 11–21.
171. Holmuhamedov EL, Jovanovic S, Dzeja PP, Jovanovic A, Terzic A. Mitochondrial ATP-sensitive K+ channels modulate cardiac mitochondrial function. *Am J Physiol* 1998; **275**: H1567–H1576.
172. De Jong JW, De Jonge R, Keijzer E, Bradamante S. The role of adenosine in preconditioning. *Pharmacol Ther* 2000; **87**: 141–149.
173. Liu Y, Sato T, O'Rourke B, Marban E. Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? *Circulation* 1998; **97**: 2463–2469.
174. Chen JC, Warshaw JB, Sanadi DR. Regulation of mitochondrial respiration in senescence. *J Cell Physiol* 1972; **80**: 141–148.
175. Hansford RG. Lipid oxidation by heart mitochondria from young adult and senescent rats. *Biochem J* 1978; **170**: 285–295.
176. Paradies G, Ruggiero FM, Petrosillo G, Gadaleta MN, Quagliariello E. Effect of aging and acetyl-L-carnitine on the activity of cytochrome oxidase and adenine nucleotide translocase in rat heart mitochondria. *FEBS Lett* 1994; **350**: 213–215.
177. Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E. Age-dependent impairment of mitochondrial function in rat heart tissue. Effect of pharmacological agents. *Ann N Y Acad Sci* 1996; **86**: 252–263.
178. Muscari C, Frascaro M, Guarnieri C, Caldarera CM. Mitochondrial function and superoxide generation from submitochondrial particles of aged rat hearts. *Biochim Biophys Acta* 1990; **1015**: 200–204.
179. Craig EE, Hood DA. Influence of aging on protein import into cardiac mitochondria. *Am J Physiol* 1997; **272**: H2983–H2988.
180. Kwong LK, Sohal RS. Age-related changes in activities of mitochondrial electron transport complexes in various tissues of the mouse. *Arch Biochem Biophys* 2000; **373**: 16–22.
181. Barja G. Mitochondrial free radical production and aging in mammals and birds. *Ann N Y Acad Sci* 1998; **854**: 224–238.
182. Muscari C, Caldarera CM, Guarnieri C. Age-dependent production of mitochondrial hydrogen peroxide, lipid peroxides, and fluorescent pigments in the rat heart. *Basic Res Card* 1990; **85**: 172–178.
183. Cusack BJ, Mushlin PS, Andrejuk T, Voulelis LD, Olson RD. Aging alters the force–frequency relationship and toxicity of oxidative stress in rabbit heart. *Life Sci* 1991; **48**: 1769–1777.
184. Lesnefsky EJ, Lundergan CF, Hodgson JM, Nair R, Reiner JS, Greenhouse SW, Califf RM, Ross AM. Increased left ventricular dysfunction in elderly patients despite successful thrombolysis: the GUSTO-I angiographic experience. *J Am Coll Cardiol* 1996; **28**: 331–337.
185. Lesnefsky EJ, Gallo DS, Ye J, Whittingham TS, Lust WD. Aging increases ischemia-reperfusion injury in the isolated, buffer perfused heart. *J Lab Clin Med* 1994; **124**: 843–851.
186. Lucas DT, Szweda LI. Cardiac reperfusion injury: aging, lipid peroxidation, and mitochondrial dysfunction. *Proc Natl Acad Sci* 1998; **95**: 510–514.
187. Frolkis VV, Frolkis RA, Mkhitarian LS, Fraifeld VE. Age-dependent effects of ischemia and reperfusion on cardiac function and Ca^{2+} transport in myocardium. *Gerontology* 1991; **37**: 233–239.
188. Liu L, Azhar G, Gao W, Zhang X, Wei JY. Bcl-2 and Bax expression in adult rat hearts after coronary occlusion:age-associated differences. *Am J Physiol* 1998; **275**: R315–R322.
189. Loeffen JL, Smeitink JA, Trijbels JM, Janssen AJ, Triepels RH, Sengers RC, van den Heuvel LP. Isolated complex I deficiency in children: clinical, biochemical and genetic aspects. *Hum Mutat* 2000; **15**: 123–134.
190. van Coster R, De Meirleir L. Mitochondrial

cytopathies and neuromuscular disorders. *Acta Neurol Belg* 2000; **100**: 156–161.
191. Tein I. Neonatal metabolic myopathies. *Semin Perinatol* 1999; **23**: 125–151.
192. Houstek J, Klement P, Floryk D, Antonicka H, Hermanska J, Kalous M, Hansikovah H, Hout'kova H, Chowdhury SK, Rosipal T, Kmoch S, Stratilova L, Zeman J. A novel deficiency of mitochondrial ATPase of nuclear origin. *Hum Mol Genet* 1999; **8**: 1967–1974.
193. Melov S, Coskum P, Patel M, Tuinstra R, Cottrell B, Jun AS, Zastawny TH, Dizdaroglu M, Goodman SI, Huang TT, Miziorko H, Epstein CJ, Wallace DC. Mitochondrial disease in superoxide dismutase 2 mutant mice. *Proc Natl Acad Sci* 1999; **96**: 846–851.
194. Sharov VG, Goussev A, Lesch M, Goldstein S, Sabbah HN. Abnormal mitochondrial function in myocardium of dogs with chronic heart failure. *J Mol Cell Cardiol* 1998; **30**: 1757–1762.
195. Ide T, Tsutsui H, Kinugawa S, Utsumi H, Kang D, Hattori N, Uchida K, Arimuraki Ki, Egashira K, Takeshita A. Mitochondrial electron transport complex I is a potential source of oxygen free radicals in the failing myocardium. *Circ Res* 1999; **85**: 357–363.
196. Quigley AF, Kapsa RM, Esmore D, Hale G, Byrne E. Mitochondrial respiratory chain activity in idiopathic dilated cardiomyopathy. *J Card Fail* 2000; **6**: 47–55.
197. Jarreta D, Orus J, Barrientos A, Miro O, Roig E, Heras M, Moraes CT, Cardellach F, Casademont J. Mitochondrial function in heart muscle from patients with idiopathic dilated cardiomyopathy. *Cardiovasc Res* 2000; **45**: 860–865.
198. Marin-Garcia J, Goldenthal MJ, Pierpont EM, Ananthakrishnan R, Perez-Atayde A. Is age a contributory factor of mitochondrial bioenergetic decline and DNA defects in idiopathic dilated cardiomyopathy? *Cardiovasc Pathol* 1999; **8**: 217–222.
199. Buchwald A, Till H, Unterberg C, Oberschmidt R, Figulla HR, Wiegand V. Alterations of the mitochondrial respiratory chain in human dilated cardiomyopathy. *Eur Heart J* 1990; **11**: 509–516.
200. Marin-Garcia J, Hu Y, Ananthakrishnan R, Pierpont ME, Pierpont GL, Goldenthal MJ. A point mutation in the cyt b gene of cardiac mtDNA associated with complex III deficiency in ischemic cardiomyopathy. *Biochem Mol Biol Int* 1996; **40**: 487–495.
201. Kubota T, Miyagishima M, Alvarez RJ, Kormos R, Rosenblum WD, Demetris AJ, Semigran MJ, Dec GW, Holubkov R, McTiernan CF, Mann DL, Feldman AM, McNamara DM. Expression of proinflammatory cytokines in the failing human heart: comparison of recent-onset and end-stage congestive heart failure. *J Heart Lung Transplant* 2000; **19**: 819–824.
202. Yue P, Massie BM, Simpson PC, Long CS. Cytokine expression increases in nonmyocytes from rats with postinfatction heart failure. *Am J Physiol* 1998; **275**: H250–H258.
203. McTiernan CF, Feldman AM. The role of tumor necrosis factor alpha in the pathophysiology of congestive heart failure. *Curr Cardiol Rep* 2000; **2**: 189–197.
204. Gudz TI, Tserng KY, Hoppel CL. Direct inhibition of mitochondrial respiratory chain complex III by cell-permeable ceramide. *J Biol Chem* 1997; **272**: 24154–24158.
205. Degli Esposti M, McLennan H. Mitochondria and cells produce reactive oxygen species in virtual anaerobiosis: relevance to ceramide-induced apoptosis. *FEBS Lett* 1998; **430**: 338–342.
206. Richter C, Ghafourifar P. Ceramide induces cytochrome c release from isolated mitochondria. *Biochem Soc Symp* 1999; **66**: 27–31.
207. Narula J, Pandey P, Arbustini E, Haider N, Narula N, Kolodgie FD, Dal Bellob B, Semigran MJ, Bielsa-Masdeu A, Dec GW, Israels S, Ballester M, Virmani R, Saxena S, Kharbanda S. Apoptosis in heart failure: release of cytochrome c from mitochondria and activation of caspase-3 in human cardiomyopathy. *Proc Natl Acad Sci* 1999; **96**: 8144–8149.