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ORIGINS AND CONSEQUENCES OF MITOCHONDRIAL VARIATION IN VERTEBRATE MUSCLE

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■ **Abstract** This review addresses the mechanisms by which mitochondrial structure and function are regulated, with a focus on vertebrate muscle. We consider the adaptive remodeling that arises during physiological transitions such as differentiation, development, and contractile activity. Parallels are drawn between such phenotypic changes and the pattern of change arising over evolutionary time, as suggested by interspecies comparisons. We address the physiological and evolutionary relationships between ATP production, thermogenesis, and superoxide generation in the context of mitochondrial function. Our discussion of mitochondrial structure focuses on the regulation of membrane composition and maintenance of the three-dimensional reticulum. Current studies of mitochondrial biogenesis strive to integrate muscle functional parameters with signal transduction and molecular genetics, providing insight into the origins of variation arising between physiological states, fiber types, and species.

INTRODUCTION

Mitochondria use oxidative phosphorylation (OXPHOS) to produce most of the energy required by aerobic tissues. Reactive oxygen species (ROS) and heat are important by-products of mitochondrial energy transduction. Apart from their role in bioenergetics, mitochondria have many catabolic and biosynthetic responsibilities in eukaryotic cell biology. It is now well known that general and specific mitochondrial defects contribute to the pathology of numerous cardiovascular (1) and neuromuscular diseases (2). Quantitative and qualitative variations in mitochondria are also important elements of both physiological and evolutionary adaptations in cardiovascular and locomotor strategies (3–5).

In this review, we summarize recent studies that address the mechanisms by which mitochondrial variation arises, focusing on the role of mitochondria in muscle energetics. Acute regulation of mitochondrial pathways allows organisms to

meet short-term changes in energy demand. When changes in metabolic demand persist for long periods, animals are able to remodel mitochondrial structure and function to better accommodate energetic needs. Remodeling of organelle and enzyme properties is integrated into developmental and physiological transitions including myogenesis, development, exercise, and environmental stress (3–5). Interspecies comparisons reveal diverse patterns of mitochondrial variation that arise through evolutionary processes. Our goal is to identify the pathways that are responsible for mediating changes in mitochondrial structure and function to provide mechanisms by which both physiological and evolutionary variation arise. As we move toward a unifying theory of mechanisms of mitochondrial adaptation, it is clear that establishing links between energetics and respiratory gene expression is of critical importance.

OXIDATIVE PHOSPHORYLATION AND ENERGY METABOLISM

Regulation of Energy Metabolism

Skeletal muscle and other aerobic tissues obtain most of their energy from mitochondrial OXPHOS (Figure 1). Control of mitochondrial respiratory activity and ATP synthesis is shared by many elements of OXPHOS (6–8). Resting muscle demonstrates low rates of both ATP synthesis and oxygen consumption. This can be attributed primarily to low concentrations of free ADP (ADP_f) and/or phosphate (9). In active muscle, ATP hydrolysis by myofibrils and ion pumps increases levels of ADP_f and phosphate. These increases stimulate Complex V, which dissipates Δp, and allow more electron transport and oxygen consumption. When mitochondria are isolated and incubated in vitro, these respiratory rates can be assessed using polarographic measurements of oxygen consumption (9). State 4 is the respiration rate exhibited by isolated mitochondria in the presence of carbon substrates, but in the absence of ADP_f. The maximal respiration rate (State 3) is induced when the system is presented with excess ADP_f and phosphate. In vivo, muscle mitochondria probably never experience the kinetic conditions that would allow them to reach State 3, the maximal respiration rate (10). Superimposed upon adenylate regulation of OXPHOS are numerous fine controls. For example, the phosphocreatine shuttle can influence the ability of adenylates to mediate changes in OXPHOS (7, 8). Changes in NADH production can arise through regulation of Ca^{2+}-sensitive mitochondrial enzymes (11) and metabolic fuel availability (8). Alterations in proton conductance are brought about by various leak pathways (12), including uncoupling proteins (13, 14). In addition, the utilization of oxygen as a substrate in the COX reaction is influenced by oxygen availability, which is determined by blood flow and oxygen gradients (15). The levels of nitric oxide also influence oxygen binding by COX (16). Several OXPHOS complexes are subject to allosteric and covalent regulation of catalytic properties mediated by ATP/ADP_f ratios (17–19). Recent studies have shown that

OXPHOS complexes are organized into supercomplexes (20, 21), which may introduce a new level of regulation that is superimposed upon metabolite regulation of OXPHOS. The relative importance of each of the various regulators of mitochondrial activity is vigorously debated, but it is widely recognized that the regulatory relationships differ in relation to metabolic rate and vary across muscle fiber types.

Vertebrate muscle also controls the balance between the oxidation of carbohydrate and lipid by regulating the type of fuels available to mitochondria, as well as the transport and oxidation of those fuels within mitochondria (22–25). Hormonal conditions, plasma metabolite levels, and cytosolic processes influence the profile of fuels available for mitochondrial uptake. In vertebrate muscle, cytosolic pyruvate concentrations saturate the monocarboxylate transporter, thus mitochondria are constantly supplied with adequate pyruvate to support State 3 respiration (10). When fatty acids become available, the balance between pyruvate and lipid oxidation is determined by the regulation of carnitine palmitoyl transferase I and pyruvate dehydrogenase (23–25). Discrimination between carbohydrate and fatty acid oxidation is dependent upon metabolic rate (10). Mitochondria from cardiac muscle and oxidative skeletal muscles (e.g., mammalian type I, fish red muscle) possess enzymatic and metabolic profiles that allow utilization of either pyruvate or fatty acids. In contrast, glycolytic fibers (mammalian type 2A, 2B, 2D/X, fish white muscle) possess mitochondria that demonstrate properties suggesting a predisposition toward carbohydrate oxidation (10, 26–28).

Although fuel selection of muscle mitochondria is reasonably conserved across vertebrates, wider phyologenetic comparisons reveal greater plasticity (29). One exception to this typical vertebrate pattern of fuel selection appears in Chondrichthyes (sharks, rays, ratfish). Muscle mitochondria from these primitive fish lack the capacity to oxidize fatty acids directly, apparently relying upon hepatic ketogenesis to provide the lipid fuel for muscle activity. The genetic basis for the peculiarities of chondrichthian muscle lipid oxidation has not been established. Although these fish lack carnitine palmitoyltransferase, some other enzymes of fatty acid β-oxidation (e.g., β-hydroxyacylCoA dehydrogenase) exist at normal activities (30). For the most part, vertebrate muscle mitochondrial fuel selection is determined by extra-mitochondrial processes that influence the availability of substrates.

Membrane Leakiness, ROS Production, and Thermogenesis

The observation that mitochondria consume small amounts of oxygen in the complete absence of ADP is usually attributed to proton leak (4). Protons crossing the mitochondrial membrane dissipate Δp, inducing a very low level of respiration to replenish the proton gradient. Although membranes in general are relatively impermeable to protons, even pure phospholipid preparations show some permeability to protons (31). Membrane phospholipid composition, which changes in response to environmental stress, can correlate with leak kinetics (32, 33). However, direct manipulation of the phospholipid profile does not seem to affect leak

rates (31). Nonspecific leak across the inner mitochondrial membrane is enhanced by the presence of integral proteins (34). Many mitochondrial transport processes require proton compensation, which also contributes to dissipation of the proton gradient (35). Thus the mechanistic basis of mitochondrial proton leak is dependent upon many factors (34). Proton leak is greatest at the highest membrane potential, particularly in the presence of succinate or other flavin-linked substrates. Interestingly, H^+ leak is linearly dependent upon membrane potential until the membrane potential exceeds a threshold value. Above this threshold value, the leak increases in a nonlinear manner, reflecting nonohmic conductance (36).

Mitochondrial proton leak is significant for several reasons. First, it prevents mitochondrial membranes from undergoing electrical collapse associated with excessive field strength. Second, because the production of ROS is greatest at the highest Δp, the leak may also serve to reduce oxidative stress (37). At rest, leak respiration may be a significant component of resting metabolic rate and heat production (38, 39). Thus the relationship between the Δp and ATP synthesis is intertwined with the role of mitochondria in production of heat and ROS.

Heat is released as a result of unavoidable inefficiencies in the process of electron transport. In any given system, mitochondrial heat production should increase in relation to respiration rate. In order to reach comparable levels of ATP synthesis, systems with higher inherent leakiness will require higher respiration rates and produce more heat as a by-product. The leakiness of homeothermic mitochondria is greater than poikilothermic mitochondria, although the reason for this is not yet established (33, 39). Uncoupling proteins (UCPs) are expressed by all eukaryotic organisms and appear to increase proton conductance (40). It is clear that brown adipose tissue uses UCP-1 to generate heat (13, 14, 41). It had long been thought that UCP-1 acted as a proton channel (14), but recently Garlid et al. (13) proposed an alternative model whereby increases in proton conductance arise through futile cycling of fatty acids between membrane layers. Specifically, uncharged fatty acids are transferred from the outer leaflet of the mitochondrial membrane to the inner leaflet, where the fatty acid deprotonates, thus acidifying the matrix. The functions of other isoforms of UCP are not yet clear, although several have been shown to increase proton conductance (34). These isoforms may not have a central role in thermogenesis, but rather function to prevent excessive Δp and participate in regulation of glucose and lipid metabolism (34, 42).

The mitochondrial electron transport system can also be an important source of ROS, specifically superoxide (43). The risk of losing electrons depends upon the redox state of the carriers. ROS production is highest when the electron transport system is reduced, which occurs at low respiratory rates (37). Mitochondria also possess vigorous anti-oxidant defenses, including anti-oxidant enzymes (Mn-superoxide dismutase, glutathione peroxidase) and free radical scavengers (e.g., cytochrome *c*, thioredoxin, tocopherol) (5). Thus the net release of mitochondrial ROS depends upon the factors that influence both production and scavenging.

The retrograde model of mitochondrial-nuclear communication suggests that mitochondrial ROS production can regulate nuclear gene expression via

ROS-sensitive signaling pathways (5, 44) (discussed below). Under some conditions, ROS production could indicate a pathological situation that would benefit from genetic compensation. Mitochondrial superoxide production is exacerbated by defects in electron transport chain stoichiometries, such as are seen in neuromuscular diseases (45–47). Whether ROS-dependent signaling is a tenable explanation for other situations, such as the hypermetabolic challenge presented by exercise and exercise training, is unclear. Acute exercise is known to produce an increase in ROS (48); however, an increase in the mitochondrial respiration rate reduces ROS production (37). Although this is a paradox for proponents of ROS-dependent signaling in retrograde control of respiratory genes, the impact of oxygen tension is not yet clear. Oxygen limitations would tend to reduce OXPHOS redox state, which should increase the propensity to generate ROS, but hypoxia may also directly affect the vulnerability of the complexes to oxidation by molecular oxygen. The effects of oxygen on OXPHOS and ROS production are difficult to evaluate in vivo because of additional uncertainties regarding intratissue oxygen gradients. Thus the potential role of ROS in mediating mitochondrial variation in vertebrate muscle remains enigmatic.

Evolutionary and Phenotypic Variation in OXPHOS

The complexes of OXPHOS are the building blocks of mitochondrial energetics. In most systems, OXPHOS complexes exist in fixed stoichiometries of approximately 1:1:3:[6–7]:[3–5] (I:II:III:IV:V) (20). Mutations in structural genes or assembly proteins lead to pathological defects in OXPHOS complexes (49). Although it would be unwise to ignore adaptive variation in the structure of these building blocks, there are relatively few examples from vertebrates where natural (nonpathological) variation in respiratory genes demonstrably alters mitochondrial structure or function. Several OXPHOS complexes possess tissue-specific isoforms of subunits, but for the most part little is known of the impact of isoform switching strategies (50). Current analyses undoubtedly underestimate the nature of variation within species (allelic variation) and between species (homologues) (51, 52). It is difficult to assess the impact of variants because of the structural complexity of the OXPHOS complexes. For instance, Complex I is notoriously difficult to assay (53) and is composed of more than 40 subunits, many with unknown function (54).

Many studies in cardiovascular and neuromuscular pathophysiology implicate OXPHOS defects in the etiology of disease. The challenges of identifying the genetic basis of respiratory diseases prompted the development of a genomic screening strategy called cybrid analysis. In this approach, mitochondria-deficient cells are fused with enucleated cells to create a cytosolic hybrid possessing a nucleus and mtDNA from different donors. Cybrid analyses have been used to support a role for mtDNA mutations in the neurodegenerative diseases (45). The OXPHOS complexes synthesized by cybrids demonstrate impaired electron flow and accelerated ROS production (45, 46). Although these studies have strengthened

the links between mitochondrial dysfunction and disease, they are considered only the first step in linking genetic variation to structural models of OXPHOS dysfunction.

Cybrid studies have also addressed the evolution of respiratory genes. Respiratory function of human cells deficient in mtDNA can be restored by transfection with mtDNA from chimpanzees and gorillas, but mtDNA from other primates is incompatible with the human nuclear background (55). The nature of the structure of multisubunit complexes imposes constraints upon evolutionary variation. Mutations that could conceivably impart favorable catalytic properties must not induce structural changes that jeopardize the ability of the subunit to form the appropriate interactions with other subunits or catalytic partners. Xenomitochondrial cybrids demonstrate that mutations in mtDNA can endow subunits with properties that allow proper function, but only in combination with the nuclear gene products of the same species. In many cases, variation in one respiratory gene is accompanied by a corresponding change in the structure of its binding partner (e.g., COX subunit II and cytochrome *c*) (56–59). Such examples of coevolution of nuclear and mitochondrial genes demonstrate the importance of maintaining the structural relationships of OXPHOS proteins. Whether such evolutionary variation improves OXPHOS function has yet to be established. Studies in neurodegenerative diseases have shown that defective complexes can accelerate cytotoxic ROS production (47). Presumably, ROS production by maladaptive mitochondrial variants would be a strong selective pressure, contributing to the conservation of mitochondrial design throughout vertebrate evolution.

Many studies assessing the evolution of mitochondrial enzyme structure and function focus on COX (Figure 2). This enzyme is frequently shown to possess considerable metabolic control (60). COX kinetics are reasonably well understood, although recent studies of allosteric regulation by ATP suggest more complexity than had long been thought to exist (17–19, 61). COX possesses both nuclear- and mitochondrial-encoded subunits and therefore reflects the constraints upon genome coordination. A variety of genetic and disease models has contributed to our understanding of the mechanisms involved in COX synthesis and degradation (Figure 2). The availability of a crystal structure now facilitates modeling of its mode of action (62).

Multiple isoforms exist for several COX subunits, including COX VIII, VIIa, VIa (50), and possibly COX IV (63). Tissue distribution studies have led to their classification as liver versus heart/muscle isoforms. Although mature muscle expresses muscle isoforms, undifferentiated muscle expresses liver isoforms. Because the exact role of these subunits has not been established, the impact of isoform switching is also unclear. It is not yet known at what point in vertebrate evolution genes for COX VIa, VIIa, and VIII duplicated and diverged. Phylogenetic analyses suggest that the COX VIa gene underwent duplication approximately 240 million years ago (56). The only COX VIa homologue identified in fish has a sequence that is equally divergent from both the liver and muscle isoforms of mammals (64). Based on its ability to alter COX H^+/e^- ratios in response to

ATP, COX VIa isoform divergence has been suggested to be associated with the development of endothermy (56, 58). In the case of COX VIII, most mammals possess two isoforms, but in humans, the heart isoform has become a pseudogene (58). The COX VIII isoform distribution between tissues and species, in combination with very high rates of evolutionary variation, suggests little functional selection.

In a series of studies, Grossman and colleagues assessed the patterns of variation in COX genes in relation to primate evolution (56–59). Variation in COX genes was categorized as synonymous if it occurred in noncoding regions or did not result in a change in amino acid (a silent mutation). With COX I, COX VIa, and COX VIIa, the rates of synonymous and nonsynonymous substitutions are similar, suggesting little positive selection of these genes in primates. However, both COX II and COX IV demonstrate much higher rates of nonsynonymous substitutions, suggesting positive selection (58). Changes in COX II, particularly in the cytochrome *c* binding domain, may have important functional consequences (58). In addition, recent evidence points to COX IV as a major site of COX catalytic regulation in relation to ATP and phosphorylation control (19).

REGULATION OF ORGANELLE STRUCTURE

The metabolic properties of muscle mitochondria depend upon active regulation of organelle structure. Recent studies have invalidated the traditional textbook image of mitochondria as collections of individual oblong organelles of similar size with sheet-like baffles of cristae extending from the inner membrane. Mitochondria in most tissues exist as a dynamic network, constantly undergoing fission and fusion (Figure 3). The mitochondrial reticulum of striated muscle penetrates the myofibrillar network and extends throughout the cell, mounted on myofibrils and other cytoskeletal elements. Electron tomographical analyses show cristae as collections of structures ranging from tubes to lamellae (65). Ultrastructural strategies are thought to be important components of both evolutionary and phenotypic variation. However, the impact of these new models on structure-function relationships has not yet been realized.

Ultrastructural Analyses of Mitochondria

Adaptive strategies that culminate in increased oxidative capacity must coordinate control of enzyme synthesis with ultrastructural changes. Ultrastructural analyses of muscle mitochondrial properties typically assess volume density (proportion of fiber volume occupied by mitochondria) and cristae density (inner membrane surface area per volume mitochondria) (66). In most vertebrate muscle, each milliliter of mitochondria has a maximal respiration rate of 3–5 ml O_2 min^{-1} and possesses approximately 20–40 m^2 of cristae surface area. In comparisons across species and fiber types, the most variable mitochondrial parameter appears to be volume density. In all vertebrate species, the most oxidative muscle type is

cardiac muscle, in which approximately 30–50% of the muscle volume is devoted to mitochondria. Mitochondrial content of tetrapod skeletal muscle fibers ranges from 1 to 10%. Fish possess a much greater range in mitochondrial content across fiber types, typically exhibiting an approximate fivefold difference between red and white fibers. Fish red muscle mitochondrial volume density approaches or exceeds that of the heart (4).

Strategies to increase the specific activity of mitochondria ($V0_2$ ml^{-1}) are somewhat limited because OXPHOS stoichiometries are generally conserved. The inner membrane of mitochondria is approximately 80% protein, primarily made up of OXPHOS complexes. This high protein content precludes significant increases in OXPHOS content per unit of membrane. Thus the catalytic scope of OXPHOS is reasonably similar across vertebrates. Muscle mitochondria demonstrate densely packed and highly organized cristae, which in the context of current tomography-based models (65), probably reflects a high proportion of lamella. Modest increases in mitochondrial-specific activity can be achieved by increasing cristae packing. Several "elite athletes" among vertebrates, including hummingbirds (67), pronghorn antelope (68), and skipjack tuna (28), have two to three times more mitochondrial cristae surface area per milliliter mitochondrial volume than the typical mammalian quadraped. Although high cristae densities are a property of insect flight muscle, it is a strategy that is relatively rare among vertebrates. It has been argued that such a high cristae packing density has the potential to impair metabolic efficiency by constraining processes that require diffusion through the matrix (66).

These ultrastructural constraints on mitochondrial variation have been recognized for some time, but very little is known about the mechanistic determinants of cristae design. Although all striated muscles possess mitochondria with extensive cristae, some cells have a simple, unfolded inner membrane. Studies with yeast mutants demonstrate that the production of cristae depends upon several proteins, including outer membrane proteins (69) and Complex V subunits (70). If these proteins are lost through deletion mutations, the inner membrane is unable to form tubular cristae, which suggests that these proteins may seed or organize the cristae. However, control of membrane biosynthesis probably determines the extent of the cristae network. High cristae densities may arise from unabated inner membrane biosynthesis, in combination with restrictions on outer membrane volume. Changes in mitochondrial structure and function, arising during phenotypic adaptation or through evolutionary selection, must be mediated through the concerted regulation of protein profiles, membrane biosynthesis, and reticulum dynamics (see below).

Membrane Biogenesis

Mitochondrial growth and remodeling require the addition of phospholipids. Almost all mitochondrial phospholipids of the inner and outer membranes are synthesized in the ER. Mitochondrial inner membranes catalyze the conversion of phosphatidylserine (PS) to phosphatidylenthanolamine (PE) via PS decarboxylase.

Cardiolipin synthesis also occurs in the inner mitochondrial membrane. Phospholipids are transferred to the mitochondria from juxtaposed regions of the ER called mitochondria-associated membranes (MAMs) (71, 72). PS is transferred from MAMs to the mitochondrial outer membrane by an unknown pathway that appears to be protein mediated and energy independent (72, 73). PS is transferred at contact sites between the outer and inner membranes and is decarboxylated by PS decarboxylase. The resulting PE can be transferred back to the ER at MAMs, where at least part of it is methylated to form phosphatidylcholine (72). Although the pathways of phospholipid synthesis and export to mitochondria are established, relatively little is known about the processes that maintain distinct phospholipid profiles in the outer versus inner membranes.

Mitochondrial inner membranes are rich in cardiolipin, a highly acidic and hydrophobic phospholipid thought to be essential for the function of many mitochondrial proteins and processes. For example, COX readily recruits cardiolipin from mixed phospholipid membranes, accumulating three to four cardiolipin molecules per COX monomer (74, 75). Studies in yeast have shown that a mutation in the cardiolipin synthase gene impairs growth, mitochondrial protein import, maximum respiratory rate, mitochondrial membrane potential, and activities of ATPase and cytochrome oxidase (76, 77). In addition, variations in cardiolipin levels are evident when comparing mitochondrial populations (78) and muscle fiber types (79) and as a result of different physiological and developmental conditions. For example, changes in cardiolipin levels occur as a result of altered thyroid status (80), chronic contractile activity (81) and, possibly, aging [(82) but see (83, 84)]. Its functional importance is suggested by the effects of adriamycin, which interacts with cardiolipin, on translocation of precursor proteins into the matrix (85, 86). In addition, a reduced affinity of cardiolipin for cytochrome *c* has been implicated in the release of this proapoptotic protein from its binding site on the outer surface of the inner membrane (87).

Modulation of lipid profiles is frequently implicated as part of the compensatory strategies associated with exercise, diet, disease, and environmental conditions. Phospholipid profiles have the potential to alter membrane fluidity and thereby the movement of membrane proteins and mobile electron carriers. Membrane remodeling can also alter permeability to protons and the catalytic properties of enzymes. Thus studies addressing the remodeling of cell membranes may have direct relevance to mitochondrial adaptation (71). Cell membrane remodeling in response to temperature is well understood. A reduction in temperature typically leads to an increase in the relative abundance of unsaturated fatty acids in phospholipid profiles as part of the homeoviscous strategy. In part, this shift is achieved through introduction of double bonds into existing fatty acids via stimulation of desaturase activity. Recent studies in prokaryotes have identified two-component systems that control desaturase gene expression in response to temperature (88). However, challenges remain in linking membrane structural changes to mitochondrial functional changes. There are few direct demonstrations that subtle remodeling of mitochondrial membrane composition impacts upon mitochondrial enzyme function.

Mitochondrial Reticulum

The organization of mitochondria into a reticulum is thought to have many benefits for aerobic tissues such as muscle (89). If the reticulum operates as an electrical cable, electrogenic events in one location could rapidly be conducted throughout the cellular network (90). Of course, this electrical conductance could also imply a global vulnerability to depolarizing events. The mitochondrial network is also thought to facilitate oxygen diffusion, particularly in muscles devoid of myoglobin (91). If the reticulum is dynamic, constantly undergoing fission and fusion, this feature would preclude the regional accumulation of defective mitochondrial proteins and DNA. However, at some point excessive defects must be selectively removed, via fission proteins, and targeted to the quality control apparatus as part of the normal process of mitochondrial turnover (92).

Many of the important regulators of mitochondrial structure have been identified. Mitofusins (Mfn) are the vertebrate homologues of *Drosophila* fuzzy onion, a GTPase located in the outer mitochondrial membrane that mediates mitochondrial fusion. Mfn1 is expressed in most tissues, but Mfn2 is expressed primarily in striated muscle (93). In comparison, dynamin-related proteins (Drp), which are distributed throughout the cell, are required for mitochondrial fission (94). Thus the mitochondrial reticulum is constantly undergoing remodeling, with the balance determined by the relative activities of Mfn and Drp (92).

The existence of a mitochondrial reticulum conflicts with the generally accepted concept of discrete subcellular populations of mitochondria. A snapshot in time would reveal the presence of two intracellular populations of mitochondria within both skeletal and cardiac muscles. Subsarcolemmal mitochondria are dispersed beneath the muscle cell membrane. Intermyofibrillar mitochondria penetrate the myofibrillar apparatus. Depending upon the taxa and fiber type, 5 to 40% of muscle mitochondria are subsarcolemmal (66). The advantage of two distinct, specialized mitochondrial populations may relate to their location with respect to metabolite production within the cell and gradients of O_2 and blood-borne metabolites in relation to capillaries. Benefits with respect to intracellular location would be expected to accrue even if the two mitochondrial populations were identical in vivo. However, biochemical differences between these two populations emerge when the mitochondria are isolated and studied in vitro (3, 4). In general, intermyofibrillar mitochondria have a higher VO_2, a greater capacity to oxidize fatty acids, and higher rates of protein import. In addition, the labilities of the two mitochondrial subpopulations differ. Although lower in total abundance, subsarcolemmal mitochondria appear to change more rapidly in response to alterations in either muscle use or disuse (3, 95). Whether this reflects a proximity to peripheral nuclei, or perhaps localization near capillaries, remains to be established. Recent studies in vivo have shown that functional and morphological heterogeneities occur in vivo in many cell types (96). Distinct populations within a single fiber could also originate by differential processing of existing organelles, rather than from intracellular differences arising during biogenesis [see (3, 4) for more discussion].

Both the maintenance and remodeling of mitochondria require pathways for protein and organelle degradation (92, 97). Mitochondrial quality control is mediated by intra-mitochondrial proteases, as well as by organellar degradation via autophagy involving lysosomal/endosomal pathways. Both pathways are likely involved in organellar maintenance. Proteolysis, by a spectrum of ATP-dependent mitochondrial proteases (e.g., Lon, m-AAA, Yme1p), facilitates the degradation of excess subunits and damaged proteins, possibly allowing the reassembly of complexes from recycled and new subunits (97). These proteases are primarily associated with organellar maintenance, specifically targeting damaged or unassembled peptides. Additionally, autophagosomes collect organelles targeted for degradation, then fuse to lysosomes, a process that is important in clearing the cell of damaged, depolarized mitochondria (92, 98). The three-dimensional structure of the mitochondrial reticulum depends upon control of fission and fusion and on the interactions between mitochondria and the cytoskeletal proteins. Several outer membrane proteins have been identified as critical for maintenance of the mitochondrial network in yeast (100). One of these proteins, Mmm1p, is an outer membrane protein that also interacts with mtDNA aggregations (nucleoids) (69). Regardless of the mechanism by which mitochondrial regions are liberated from the reticulum, mitochondrial organelles destined for autophagocytosis must first undergo depolarization, which would be expected to accompany mitochondrial damage (98). In addition to its role in clearing damaged mitochondria, this pathway is also expected to be important under situations where reductions in mitochondrial content are required as part of phenotypic adaptation. Active reduction of mitochondria is seen in skeletal muscle in response to detraining (3) and in cardiac muscle undergoing left ventricular regression in response to anti-hypertensive treatment (99). One mechanism by which depolarization can be induced in otherwise normal mitochondria is through the induction of the mitochondrial permeability transition (98). However, its role in modifying the mitochondrial reticulum structure during decreases in mitochondrial content remains to be established.

REGULATION OF MITOCHONDRIAL CAPACITY

Mitochondrial Remodeling in the Differentiation and Development of Muscle

Muscle energy demand changes throughout the lifetime of the organism, and muscles are able to remodel energetics accordingly. Prior to differentiation, myoblasts possess mitochondria that contribute approximately 40% of the ATP for energy metabolism (101). Myogenesis is initiated by a change in hormonal conditions, including autocrine stimulation by IGF-II and deprivation of, or desensitization to, bFGF and TGF-β (102). Mitochondrial proliferation and remodeling are integrated into this myogenic program during which mitochondrial enzyme activities increase severalfold (103). However, during the rapid early phases of myogenesis,

the quantitative relationships between enzymes and mitochondrial ultrastructure can change with different time courses, implying that mitochondrial compositional changes can arise during differentiation (103). For example, activities of selected matrix enzymes increase fivefold when mitochondrial volume density increases by only 50%. Cristae surface area and cristae enzymes increase in parallel, but to a lesser extent than do matrix enzymes (103). During differentiation, mitochondria fuse into a more continuous reticulum (104). There is also a switch in tissue-specific isoforms of OXPHOS subunits and other mitochondrial enzymes (50). These mitochondrial changes coincide with a shift in energy metabolism from glycolytic to oxidative pathways, but the overall metabolic rate does not change in response to myogenesis (101). The links between the hormonal induction of myogenesis and remodeling of mitochondria have not yet been widely explored, although alterations in mitochondrial activity appear to have a profound influence on myogenesis (105), possibly through thyroid hormone–mediated pathways (106).

After the myogenic program is complete, muscles retain a modest capacity for remodeling in response to changes in activity levels. Reductions in muscle activity brought about by immobilization (107), denervation (79), or microgravity (108) reduce mitochondrial content. Conversely, a program of regularly performed endurance exercise, which considers the important training parameters of exercise intensity, duration, and frequency per week, can lead to mitochondrial reticulum expansion (3). This adaptation is particularly evident in the subsarcolemmal regions of the muscle cell, and these alterations have profound metabolic effects during acute exercise. During muscle contraction, the rise in ADP_f concentration stimulates glycolysis and drives the creatine phosphokinase reaction to consume phosphocreatine and form ATP. Because endurance training increases the mitochondrial content of skeletal muscle without large effects on creatine phosphokinase or glycolytic enzymes, a greater fraction of the energy requirements of muscle contraction is derived from mitochondrial respiration. This observation, established in vivo (109, 110), has been attributed to a greater sensitivity of mitochondrial respiration to ADP_f because a lower concentration of the metabolite is required to attain the same level of oxygen consumption. This reduction in ADP_f leads to reduced rates of glycolysis, lactic acid formation, and the utilization of phosphocreatine. In addition, a lower rate of AMP formation will occur, leading to reduced ammonia and inosine mono-phosphate synthesis in fast-twitch muscle, as well as a diminished conversion of AMP to adenosine in slow-twitch muscle. Coincident with these changes are increased activities of mitochondrial β-oxidation enzymes as a result of endurance training (111), which predisposes the individual toward greater lipid oxidation, rather than carbohydrate oxidation, during exercise. This effectively spares the limited carbohydrate resources (i.e., liver and muscle glycogen) at the expense of more abundant lipid stores (112). In large measure, these energetic adaptations form the basis for the marked improvements in muscle endurance performance observed in humans and other animals.

Thus muscle mitochondrial content is established during differentiation and development, then maintained or modified through the lifetime of the animal. In

the following sections, we discuss the constitutive and inducible pathways that are responsible for obtaining the appropriate mitochondrial content in muscle.

Control of Mitochondrial Gene Expression

Until recently, the control of expression of nuclear-encoded respiratory genes (Figure 4) had been studied primarily from the perspective of promoter analyses (3–5). Apart from the typical control elements characteristic of constitutive expression, several respiratory genes were shown to possess consensus regulatory sequences for the transcription factors nuclear respiratory factor (NRF)-1 and NRF-2 (113). NRF-1 sites also exist in the promoters of regulators of mtDNA replication and transcription, particularly mitochondrial transcription factor A (Tfam), as well as δ-aminolevulinate synthase (ALAs), the rate-limiting enzyme of heme synthesis (113). However, mitochondrial genes involved in fatty acid oxidation lack NRF-1 and NRF-2 consensus sequences and appear to be regulated by members of a family of ligand-regulated nuclear receptors, called peroxisome proliferator-activated receptors (PPAR). There has been a general failure to demonstrate that adaptive changes in mitochondrial content are due primarily to changes in NRF-1/NRF-2 activities/levels; however, the impact of PPAR isoforms on COX gene expression remains unstudied.

In the past two years, attention has focused on the participation of a transcriptional coactivator, PGC-1 (PPAR gamma coactivator-1), in the control of mitochondrial biogenesis. PGC-1 interacts with a spectrum of ligand-regulated DNA-binding transcription factors such as NRF-1, PPARγ, and PPARα (114, 115). A PGC-1 related coactivator (PRC) has also been identified, with properties that overlap with those of PGC-1 (116). When transcription factors/nuclear receptors bind DNA, PGC-1 docks and undergoes a conformational change that recruits other coactivator proteins thereby increasing transcriptional activity (114). In theory, increases in mitochondrial gene expression could be achieved through upregulation of the primary transcription factor (e.g., NRFs, PPARs) or the docking coactivator (PGCs). A growing body of evidence implicates PGC-1 in the control of mitochondrial biogenesis. Certainly, increases in PGC-1 mRNA and protein levels accompany mitochondrial proliferation during adaptive thermogenesis (117) and muscle regeneration (118). Overexpression of PGC-1 leads to mitochondrial proliferation in transgenic mouse heart (119) and in adipocytes and myoblasts (114). Because it appears that PGC-1 modulates transcription of respiratory genes, attention has focused on the regulation of PGC-1 synthesis and activity (120–123). For example, PGC-1 activity can be activated by a p38 MAPK-sensitive pathway, possibly through effects on a PGC-1 repressor (114, 122). Other studies have shown p38 MAPK-dependent activation of PPARα (121). Regulation of respiratory gene expression via p38 MAPK-dependent pathways may be important in mediating mitochondrial proliferation induced by extrinsic pathways such as the response to cytokines (123). Although the role of membrane receptor signaling pathways in mediating mitochondrial proliferation in response to exercise is

largely unknown, there is considerable evidence that intrinsic pathways, dependent on muscle metabolism, also influence respiratory gene expression.

Sensing and Responding to Energy Metabolism

Cellular control over adaptive changes in mitochondrial content demands a capacity to sense the need for additional mitochondrial energy production, followed by triggering of signaling pathways that culminate in an increased and coordinated expression of respiratory genes. It is possible that adaptive changes in mitochondrial content simply reflect an increase in the activity of a constitutive pathway. Alternatively, the capacity to undergo adaptive changes may be mediated by a separate inducible pathway that could lead to alterations in mitochondrial composition and function.

Many conditions that lead to changes in bioenergetics result in mitochondrial proliferation. Although most attention focuses on the control of respiratory gene expression, it is important to recognize that other processes (e.g., mRNA stability, the post-translational modification, import, folding, assembly) contribute to the mitochondrial proliferative response (3). Despite advances in understanding the roles of transcription factors/nuclear receptors (NRFs/PPARs) and coactivators (PGC-1) in transcriptional control of respiratory genes, relatively little is known of the pathways by which these proteins are regulated. A longstanding question has been, are there metabolic signals that induce mitochondrial biogenesis? Contractile activity induces changes in adenylate concentrations, ROS production, neuronal stimulation, and calcium homeostasis, each of which has been implicated in control of expression of genes involved in bioenergetics, as well as in excitation-contraction coupling and the contractile apparatus. These metabolic controls are not necessarily independent of each other.

Several lines of evidence offer support for links between energy metabolism and transcription factor levels or DNA binding activity. Many studies have shown increases in NRF-1 in response to contractile activity. Uncoupling of HeLa cells, through expression of UCP-1, triggers expression of NRF-1 and NRF-1-sensitive genes (124). The metabolic disturbance produced by the chronic treatment of animals with β-guanidinopropionic acid, which reduces cellular phosphocreatine and ATP, led to an increase in NRF-1-DNA binding and the subsequent elevation of mitochondrial proteins and mitochondrial volume (125). This treatment causes an increased activity of AMP-activated protein kinase (AMPK). Furthermore, treatment of animals with the 5-aminoimidazole-4-carboxamide-ribofuranoside (AICAR) for four weeks, another method of increasing AMPK activity, increases at least some mitochondrial enzymes (126). Thus it seems that metabolic changes that produce an activation of AMPK may form part of the initial signaling pathway. In addition to direct sensitivity to AMP levels, AMPK levels are regulated at the transcriptional levels, and AMPK activity is regulated by AMPK-kinase (AMPKK) (127). AMPK signaling has been implicated in other pathways that demonstrate responsiveness to metabolic conditions (127). Direct links between

AMPK and other respiratory gene effectors have not yet been shown. It should be noted that this response may not be mediated solely by NRF-1 because NRF-1 sites are not universally found in all promoters of nuclear genes encoding mitochondrial proteins. This is true, for example, even within the 10 nuclear-encoded subunits of cytochrome *c* oxidase (50). In addition, not all mitochondrial enzymes are increased by AICAR treatment. Thus additional signals are likely to be important.

Numerous models have suggested that alterations in ROS production accompany physiological and pharmacological effectors of respiratory gene expression, including exercise (48). Parallel relationships between respiratory gene expression and ROS production have also been shown in cells with defective mtDNA (46) or incompatible genomes (47). Many signaling pathways have been shown to be responsive to ROS (5). Both AP-1 and NF-κB are thought to respond at many levels to ROS and oxidative stress. Several studies have shown correlations between ROS, AP-1/NFκB activities, and respiratory gene expression (128). However, few direct links between the activity of AP-1 or NFκB and expression of either respiratory genes or their transcription regulators have been established. Electrical stimulation of cardiomyocytes induces cytochrome *c* expression through effects on AP-1 signaling via c-Jun N-terminal kinase (JNK), and NRF-1 (129, 130). In this study, the link between electrical stimulation and respiratory gene expression is probably not ROS but more likely Ca^{2+}.

There are many clear links between Ca^{2+} and respiratory gene expression mediated via Ca^{2+}-dependent regulatory enzymes. Although calcineurin (CaN), a Ca^{2+}-sensitive protein phosphatase, is implicated in both the hypertrophic response (131) and muscle remodeling (132), there is little evidence that it is involved in the associated mitochondrial changes. In contrast, two Ca^{2+}-sensitive protein kinases, CamK (Ca^{2+}/calmodulin-dependent protein kinase) and protein kinase C (PKC), have been implicated in control of respiratory gene expression. The treatment of muscle cells with the Ca^{2+} ionophore A23187 to increase intracellular Ca^{2+} concentrations resulted in increases in the transactivation of the cytochrome *c* gene (133). This effect appeared to be mediated via a Ca^{2+}-sensitive, PKC-dependent pathway. Recently, transgenic mice were developed with a CamK gene that is expressed only in skeletal muscle and in a constitutively active form. Skeletal muscle of these mice demonstrated pronounced mitochondrial biogenesis, as indicated by morphological (i.e., increased subsarcolemmal mitochondrial content), biochemical (i.e., increased nuclear and mitochondrial gene products), functional (i.e., improved fatigue resistance), and molecular (i.e., augmented transcription of PGC-1) indices (134). Direct links between CamK and PGC-1 expression are not yet established, but these data suggest a role for CREB in mediating Ca^{2+}-dependent control of mitochondrial gene expression. However, it remains to be shown that this signaling operates at a sufficiently high level during contractile activity. In muscle cell culture, electrical stimulation leading to increases in cytosolic Ca^{2+}, in which contractions and ATP turnover were inhibited with the drug butanedione monoxamine, did not result in the typical increase in cytochrome

c transactivation that is produced in the absence of butanedione monoxamine (108). This result also suggests the involvement of a metabolic signal in mediating mitochondrial biogenesis, at least with respect to cytochrome *c*. Studies in muscle cells with experimentally induced mtDNA depletion support the contention that Ca^{2+} and metabolic signaling are intertwined. Depletion of mtDNA results in a reduced synthesis of ATP because of a defective mitochondrial respiratory chain, which relies on the availability of mtDNA gene products for proper function (136). This leads to an increase in cytosolic Ca^{2+} concentration, presumably because the energy-dependent processes of Ca^{2+} extrusion and uptake are impaired. An upregulation of a number of proteins related to Ca^{2+} release and responsiveness, as well as nuclear genes encoding mitochondrial proteins (i.e., COX Vb), was observed (136). The interpretations of these data are that imbalances between cellular ATP demand and mitochondrial ATP supply, leading to alterations in Ca^{2+} homeostasis, can trigger the induction of signal transduction pathways leading to the phosphorylation or dephosphorylation of transcription and/or stability factors. Thus this may be indicative of the signaling events that take place during contractile activity.

Taken together, this information tells us that multiple signaling events are involved in muscle mitochondrial biogenesis, perhaps providing redundant mechanisms to ensure that the multiple processes involved in organelle synthesis can occur.

Evolutionary Variation in Mitochondrial Capacity

Interspecies comparisons reveal many examples of variation in mitochondrial capacity arising as a result of complex evolutionary processes. Allometric comparisons show that small species possess higher specific activities of mitochondrial enzymes than do larger species. Animal physiologists are also familiar with comparisons between athletic and nonathletic species. Although we know a great deal about the molecular genetics responsible for changes in mitochondrial enzymes within individuals, the basis for differences between species is much less understood. Based on these studies within species, a few generalizations can be predicted regarding the nature of variation in mitochondrial content between species.

First, it is unlikely that the pathways used by individuals to increase mitochondrial content are exclusively responsible for establishing the differences seen between species. Athletic species (e.g., dogs) are unlikely to be simply exercise-trained versions of less athletic species (e.g., goats). Most animals possess a capacity to increase mitochondrial content through exercise training. A strategy that recruits inducible pathways to elevate constitutive expression would compromise muscle responsiveness. Garland has used artificial selection to establish lines of mice that exhibit profoundly different levels of voluntary exercise. Active lines exhibit about 20% more mitochondrial enzymes than do sedentary lines, but both active and sedentary lines elevate mitochondrial content to the same extent with

exercise training (137). Thus even population-level variation in the constitutive expression of respiratory genes does not preclude the ability to induce further increases with training. The genetic basis of population-level variation in mitochondrial content in this powerful model is unknown.

Second, evolutionary variation in mitochondrial content probably arises through altered expression of effectors of signaling pathways, rather than individual respiratory genes. Although mitochondria are extremely complex to build and maintain, increases in mitochondrial content can arise with relatively modest genetic changes (e.g., mutations, duplications). Studies using transgenic mice illustrate the potential impact of the sort of genetic variation that could arise over evolutionary time. Mice overexpressing PGC-1 (116), CamK (134), or myogenin (138) demonstrate muscle mitochondrial proliferation, supporting the generalization that broad suites of respiratory genes are controlled by a very small number of transcriptional regulators. The most parsimonious route to elevating mitochondrial content is through targeting signaling pathways (transcriptional coactivators, regulatory enzymes, transcription factors) rather than individual respiratory genes. Nonetheless, other transgenic studies show that mitochondrial proliferation and remodeling can also arise in mice with transgenes that target metabolic pathways. Knockout mice lacking muscle-specific adenine nucleotide translocase demonstrate proliferation of mitochondria, albeit with severe defects (139). Mice overexpressing muscle-specific lipoprotein lipase also show increased mitochondrial content, with enhanced capacity for fatty acid oxidation (140). Such genetic strategies would be expected to cause pleiotropic effects on metabolism.

Third, evolutionary variation that leads to altered mitochondrial content in skeletal muscle must restrict its effects to specific tissues, primarily muscle. Mutations that lead to elevated mitochondrial content in all tissues could have negative effects (e.g., oxidative stress) in nonmuscle tissues. In transgenic mouse studies, transgenes are typically constructed with muscle-specific promoters to restrict mitochondrial changes to this tissue. At the moment it is not known if the studies on transgenic mice foreshadow the sort of variation that might be revealed in future evolutionary comparisons.

With the solidification of the role of mitochondria in a broad spectrum of diseases, there has been a recent explosion in studies of mitochondrial structure and function. Future studies in comparative physiology and metabolism will build upon advances in biomedical fields to address the basis of evolutionary variation in bioenergetics, and help us understand the intricacies of organelle (i.e., mitochondrial) biogenesis.

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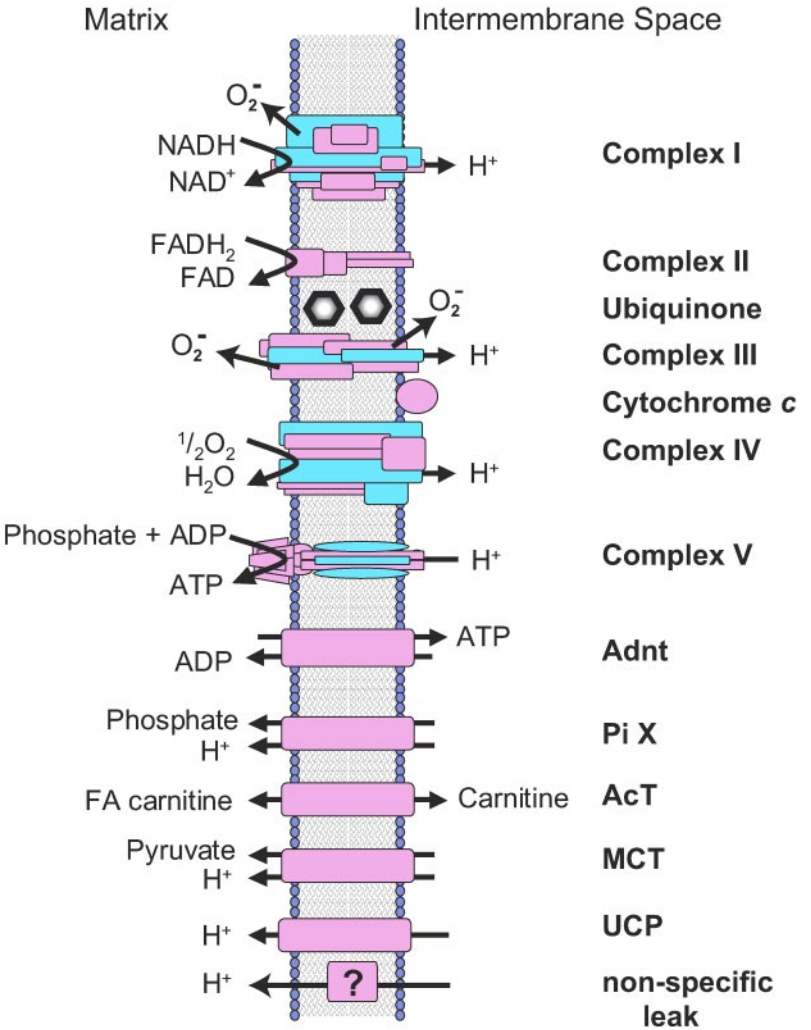

Figure 1 Oxidative phosphorylation. Reducing equivalents (NADH, $FADH_2$) are produced in mitochondria through the activity of the enzymes in the tricarboxylic acid cycle, fatty acid β-oxidation, and redox shuttles. Oxidation of reducing equivalents by Complex I (NADH) and Complex II ($FADH_2$) begins the process of electron transport. Electrons are transferred to ubiquinone, Complex III, cytochrome *c*, Complex IV, or cytochrome *c* oxidase (COX) then oxygen. During electron transfer, protons are pumped across the inner membrane, creating the proton motive force (Δp). The Δp can be used by Complex V to drive ATP synthesis, which also requires the activity of the adenine nucleotide translocase (Adnt) and the phosphate exchanger (PiX). It also provides the energy to transport ions, metabolites, and proteins into mitochondria. The monocarboxylate transporter (MCT) is a proton symport carrying pyruvate and other monocarboxylates (e.g., ketone bodies) into mitochondria. Long chain fatty acids are transported into mitochondria as carnitine esters by the acylcarnitine-carnitine translocase (Act).

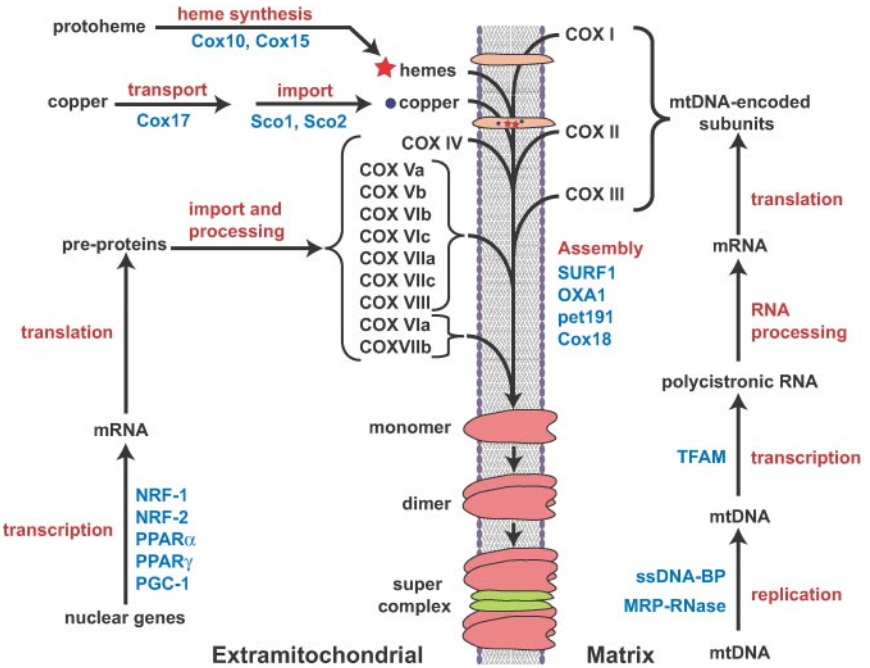

Figure 2 COX assembly. Vertebrate COX is composed of 3 mtDNA-encoded subunits and 10 nuclear-encoded subunits. Regulation of nuclear gene expression is controlled by several transcription factors (NRFs, PPARs) and coactivators (PGC-1). The import proteins (tim/tom apparatus) transfer peptides across the inner membrane into the matrix, where they are folded by the hsp70 homologue grp75. Several other nuclear gene products interact with the regulatory D-loop of mtDNA to control replication and transcription. The polycistronic nature of the primary transcript from mtDNA results in stoichiometric levels of mRNA for COX I, II, and III. Assembly of COX begins with insertion of the catalytic core subunit COX I into the inner membrane, followed by addition of hemes, metals, and other subunits. Several assembly factors critical for synthesis of COX monomers have been identified (see 49). Most COX probably exists in inner membranes as super-complexes with complex III (green), in a monomer ratio of 4:2 (21). COX can also be regulated by pathways that alter membrane properties such as cardiolipin profiles.

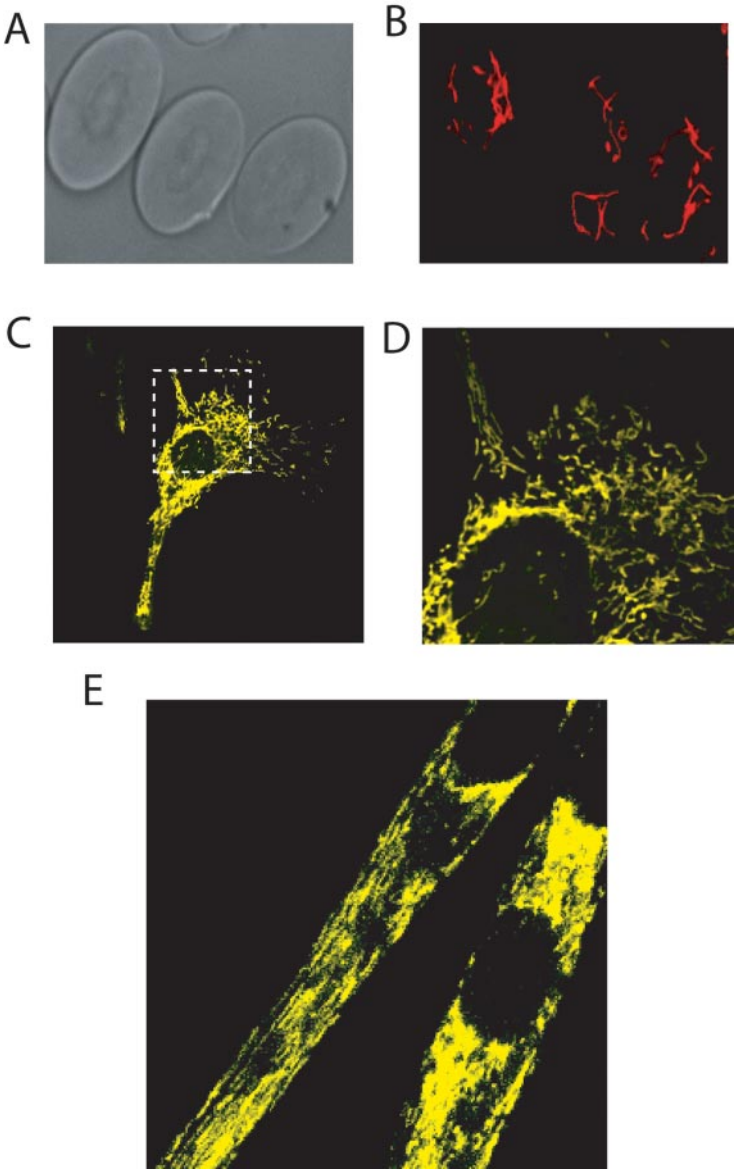

Figure 3 Mitochondrial ultrastructure. In most cells, mitochondria exists as a collection of inter-connected organelles. This reticulum can be visualized using fluorescent dyes such as tetramethyl rhodamine methyl ester that accumulate in the mitochondria in relation to the membrane potential. Trout erythrocytes (*A*) are loaded with TMRM (*B*) to visualize the mitochondrial network. The mitochondrial network can also be seen in mouse myoblasts (*C*, *D*) and myotubes (*E*) that have been transfected with a genetic construct encoding green fluorescent protein targeted to the mitochondria via signal sequence from a nuclear encoded COX gene (Images courtesy C. Lyons).

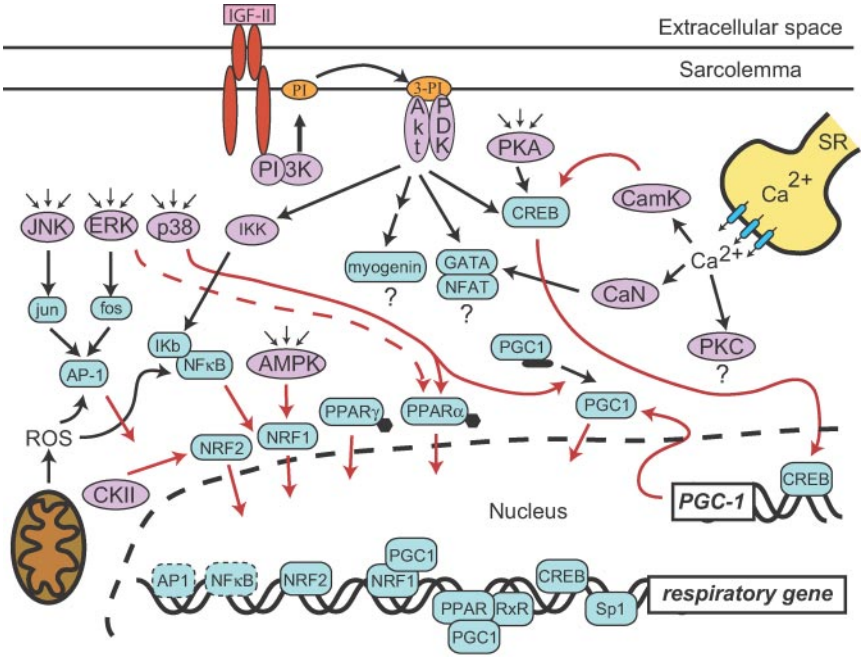

Figure 4 Control of respiratory gene expression. Nuclear-encoded respiratory genes are regulated by both constitutive and inducible pathways. Protein-modifying enzymes (kinases, phosphatase) are denoted in pink ovals. Transcriptional regulatory proteins are denoted by blue rounded boxes. Myogenesis depends upon the IGF-II dependent activation of PI3K and Akt, but the links to concomitant respiratory gene expression have not been established. Red arrows signify connections between signaling pathways and respiratory gene expression discussed in more detail in the accompanying text. The respiratory gene promoter illustrated is idealized; not all respiratory genes possess the binding elements for each transcription factor. The transcription factors AP-1 and NFκB are shown on the promoter with broken lines. Although many respiratory genes possess these consensus elements, their role in regulation of respiratory genes has not been established. See text for more detail.

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