Plasticity in Skeletal, Cardiac, and Smooth Muscle
Invited Review: Contractile activity-induced mitochondrial biogenesis in skeletal muscle

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Hood, David A. Invited Review: Contractile activity-induced mitochondrial biogenesis in skeletal muscle. J Appl Physiol 90: 1137–1157, 2001.—Chronic contractile activity produces mitochondrial biogenesis in muscle. This adaptation results in a significant shift in adenine nucleotide metabolism, with attendant improvements in fatigue resistance. The vast majority of mitochondrial proteins are derived from the nuclear genome, necessitating the transcription of genes, the translation of mRNA into protein, the targeting of the protein to a mitochondrial compartment via the import machinery, and the assembly of multisubunit enzyme complexes in the respiratory chain or matrix. Putative signals involved in initiating this pathway of gene expression in response to contractile activity likely arise from combinations of accelerations in ATP turnover or imbalances between mitochondrial ATP synthesis and cellular ATP demand, and Ca2+ fluxes. These rapid events are followed by the activation of exercise-responsive kinases, which phosphorylate proteins such as transcription factors, which subsequently bind to upstream regulatory regions in DNA, to alter transcription rates. Contractile activity increases the mRNA levels of nuclear-encoded proteins such as cytochrome c and mitochondrial transcription factor A (Tfam) and mRNA levels of upstream transcription factors like c-jun and nuclear respiratory factor-1 (NRF-1). mRNA level changes are often most evident during the postexercise recovery period, and they can occur as a result of contractile activity-induced increases in transcription or mRNA stability. Tfam is imported into mitochondria and controls the expression of mitochondrial DNA (mtDNA). mtDNA contributes only 13 protein products to the respiratory chain, but they are vital for electron transport and ATP synthesis. Contractile activity increases Tfam expression and accelerates its import into mitochondria, resulting in increased mtDNA transcription and replication. The result of this coordinated expression of the nuclear and the mitochondrial genomes, along with poorly understood changes in phospholipid synthesis, is an expansion of the muscle mitochondrial reticulum. Further understanding of 1) regulation of mtDNA expression, 2) upstream activators of NRF-1 and other transcription factors, 3) the identity of mRNA stabilizing proteins, and 4) potential of contractile activity-induced changes in apoptotic signals are warranted.

Since mitochondria are a major source of cellular ATP, we should not be surprised to learn that the number of them per cell, as well as their intracellular locations, varies with the type of cell and with its metabolic state (41).

Robert Dyson’s words, taken from his 1975 undergraduate textbook, had only recently been established. Cer-
tortly, tissue differences in mitochondrial content were well known, but it was only a few years earlier that mitochondrial size, number, and/or volume was demonstrated to increase within a given tissue (i.e., skeletal muscle) in response to a physiological stimulus (i.e., endurance training; Refs. 57, 75, 87, 95). This finding became relevant to exercise physiologists interested in the cellular basis of endurance performance, and it was of practical importance for the athletes involved in training programs. In addition, clinical relevance could be derived from the fact that mitochondrial content could also be reduced during periods of prolonged muscle disuse, such as limb immobilization (16) or muscle denervation (180). However, the broader impact of changes in mitochondrial content within a tissue to other fields of cell biology were not as apparent as they are today. Discussions of mitochondrial function, gene expression, and synthesis are now a regular feature of cell biology laboratories. The term “mitochondrial biogenesis,” implying the cellular processes involved in the synthesis and degradation of the organelle, is now used with more regularity and apparent relevance. Mitochondria are now recognized not only as “power-houses of the cell,” for which they are famous, but as potential loci of disease with broad clinical relevance (104, 176).

Our understanding of mitochondrial function has progressed rapidly in recent years for a number of reasons. First, the remarkable advances in molecular biology involving recombinant DNA techniques, quantitative analyses of DNA and RNA, the use of animal and cell culture models (189), and the advent of gene transfer technology (193) have all permitted researchers to probe more deeply into cellular and molecular events involved in mitochondrial function. These are complemented by advancements in microscopy techniques, which permit improved resolution of organelle structure and, when combined with fluorescent detection methodology, measurement of ion movements in various compartments of living cells (50). The result of this is that we now appreciate new roles for mitochondria, not only as the major ATP-providing organelle but also as mediators of apoptotic events leading to cell death, as housing for a separate, semi-autonomous genome [i.e., mitochondrial DNA (mtDNA)] and as a site of cellular signal transduction events, which may help coordinate nuclear and mitochondrial gene expression.

Mitochondrial biogenesis can be a dramatic event, particularly when it is invoked in a previously low-oxidative, white skeletal muscle. Because of this, it can also serve as a cellular model of organelle assembly within eukaryotic cells. In response to exercise, the conversion of phenotype of a white muscle to one with a visibly red appearance is brought about by additional heme synthesis and its incorporation into mitochondrial cytochromes as well as into myoglobin. As discussed below, the large increase in mitochondrial content observed can have a profound impact on cellular metabolism during the stress of exercise. This review will focus on current issues in mitochondrial biogenesis, with emphasis on skeletal muscle and the role of contractile activity (i.e., exercise). A number of other reviews have appeared on this topic (44, 76, 81, 180).

FUNDAMENTALS OF MUSCLE MITOCHONDRIAL BIOGENESIS

Mitochondrial Biogenesis Can Be Produced by a Number of Physiological Conditions

It is well known that endurance training, employing an appropriate duration per day, frequency per week, and submaximal intensity per exercise bout, can produce an increase in mitochondrial content, usually ranging from 50 to 100% within ~6 wk. This directly results in improved endurance performance, largely independent of the much smaller training-induced changes in maximal oxygen consumption (36, 48). This mitochondrial biogenesis can be replicated using artificial models of exercise, such as chronic contractile activity produced by electrical stimulation of the motor nerve. This model has the advantage of producing relatively larger changes in mitochondrial biogenesis, which can occur in a shorter time frame (1–3 wk; Refs. 43, 132, 167). Muscle mitochondrial biogenesis can also result from other physiological conditions, including thyroid hormone treatment (185). This represents a very different stimulus, acting via nuclear and mitochondrial receptors (see Expression of mtDNA, below), to ultimately produce a greater mitochondrial content. The response of muscle to thyroid hormone is fiber-type specific (185), likely dependent in part on differences in the distribution of nuclear thyroid hormone receptors (157). The independent cellular pathways employed by thyroid hormone treatment and contractile activity to produce mitochondrial biogenesis are also evident from the fact that increases in mitochondrial content are still evident in muscles of animals that have been thyroidectomized and subject to chronic contractile activity (56, 80, 171). Nonetheless, both conditions appear to result in an increased functional mitochondrial mass within muscle cells, resulting in a greater capacity for oxygen consumption per gram of tissue. The interaction and/or differences in the way these two stimuli operate to produce this effect remain, in large part, to be determined.

Mitochondrial Content Has a Short Half-Life, and Mitochondrial Phenotype Can Change

Mitochondrial content can be measured directly, using morphometric estimates of organelle volume in relation to total cellular volume. More commonly, it is estimated by the change in maximal activity, measured under optimal conditions in vitro, of a typical “marker enzyme” such as citrate synthase, or by the change in content of a single protein-like cytochrome c (170). This is valid under most conditions because changes in mitochondrial volumes estimated morphometrically parallel the changes in enzyme maximum velocity ($V_{max}$) values (141). The measurement of single proteins or enzyme activities has been useful for
determining mitochondrial turnover, assuming that the behavior of the protein resembles that of the organelle as a whole. This assumption must be considered in light of the growing recognition that mitochondrial protein composition can change in response to chronic exercise (69, 79, 129, 166), particularly when combined with a variety of conditions such as iron deficiency (64), inhibition of mitochondrial protein synthesis (115), or in mitochondrial disease produced by mtDNA deletions.

Measurements of representative protein markers such as cytochrome c or cytochrome-c oxidase (COX) activity have allowed exercise physiologists to recognize that, in response to a constant exercise stimulus, ~6 wk of endurance training is required to reach a new, higher steady-state mitochondrial content. This time course is dependent on the fiber type being recruited, as well as the duration, frequency, and intensity of the exercise bouts (39, 65, 72, 83). Mitochondrial adaptations will not occur in skeletal muscle cells, which are not recruited during the exercise bout, consistent with the idea that the stimulus for biogenesis originates within the contracting muscle, independent of humoral influences. A corollary of this is that endurance training programs must be geared, in part, toward the recruitment of fast-fatiguable motor units containing type IIb (fast-twitch white) fibers if any mitochondrial adaptation is expected in those fibers. Interestingly, resistance training, which does indeed recruit fast-fatiguable motor units, does not lead to a mitochondrial adaptation. Because the very high intensity and low duration of most resistance training regimens represent such a strong stimulus for the synthesis of myofibrillar proteins leading to muscle hypertrophy, the mitochondrial content within enlarged muscle fibers may even be “diluted” within the cell (113, 114). This increases the diffusion distance of oxygen and substrates and does not represent a favorable adaptation with respect to endurance performance.

The approximate 6-wk time period required to achieve a new steady-state mitochondrial content in response to endurance training clearly does not reflect the early molecular events that ultimately lead to the measurable morphological changes. Indeed, changes in mitochondrial protein content can be visibly apparent at much earlier time points. Mitochondrial proteins turn over with a half-life of ~1 wk after the onset of a new level of muscle contractile activity (15, 70, 73, 170). This means that a continuous exercise stimulus is required to maintain mitochondrial content at an elevated level following a training period; otherwise, loss of oxidative capacity and endurance performance will ensue. It is interesting to note that mitochondrial phospholipid content changes occur with an even shorter half-life (~4 days; Refs. 167 and 180), suggesting that the assembly and/or degradation of the organelle could be initiated by changes in phospholipid composition. In the absence of normal rates of cytochrome synthesis and incorporation into the inner membrane during iron deficiency, imposed contractile activity appears to lead to continued membrane lipid synthesis, producing an increased mitochondrial volume with a markedly reduced and abnormal protein content (64). These data indicate that phospholipid and protein synthesis are not necessarily coupled during contractile activity-induced mitochondrial biogenesis. The data also suggest that the ratio of lipid to protein within mitochondria must change somewhat during the transition to either higher or lower mitochondrial contents. This change appears to be subtle as a result of training (57) or chronic stimulation (141) and in most cases appears not to affect rates of oxygen consumption per unit volume of mitochondria (85, 86). However, it may be much more evident under steady-state conditions in tissues possessing widely divergent mitochondrial contents, such as heart and white skeletal muscle (180).

**Mitochondrial Biogenesis Leads to Changes in Cellular Metabolism and Muscle Performance During Endurance Exercise**

The marked improvement in endurance performance that results from mitochondrial biogenesis is a consequence of changes in muscle metabolism during exercise. During acute muscle contraction, the concentration of free ADP (ADPf) rises. This increase drives the creatine phosphokinase (CPK) equilibrium reaction toward the formation of ATP and creatine (Fig. 1). ADPf is also a substrate and allosteric activator in the glycolytic pathway, and it controls active (state 3) mitochondrial respiration. Because endurance training increases the mitochondrial content of skeletal muscle without large effects on CPK or glycolytic enzymes, it seems reasonable to assume that a greater fraction of the energy requirement of a given work effort will now be derived from aerobic metabolism. This has been referred to as a greater sensitivity of mitochondrial respiration to ADPf since a lower increase in the concentration of the metabolite is required to attain the same level of oxygen consumption (Fig. 1; Refs. 31 and 40). This reduced increase in ADPf will attenuate glycolysis and the formation of lactic acid, and a sparing of phosphocreatine will result. In addition, a lower rate of AMP formation will occur. In fast-twitch muscle with a high-AMP deaminase activity (172), ammonia and IMP synthesis will be reduced. In slow-twitch muscle possessing lower AMP deaminase and higher 5'-nucleotidase activities, the lower formation of AMP will be translated into reduced conversion to adenosine (Fig. 1). Along with the smaller decline in creatine phosphate, a reduced synthesis of AMP will lead to a lower activation of AMP kinase (AMPK; Ref. 186), a factor that may be important for the training-induced reduction in GLUT-4 translocation from the endosomal pool to the plasma membrane (143). This, coincident with increased activities of mitochondrial β-oxidation enzymes (121), predisposes the individual toward greater lipid oxidation during exercise.

The increased mitochondrial content brought about by endurance training not only leads to a reduced lactate production but also enhances the disposal of
lactate. Recently, lactate dehydrogenase has been localized within the organelle (20), and lactate transport into mitochondria can occur via the mitochondrial monocarboxylate transporter MCT1 (19). A higher mitochondrial content as a result of chronic exercise will therefore improve lactate oxidation, resulting in lower rates of release from muscle (38). In addition, the higher the mitochondrial content per gram of muscle, the higher the rate of respiration required per mitochondrion for any given workload (Fig. 1, bottom). This has been predicted to reduce the level of potentially damaging reactive oxygen species (ROS; Ref. 36), even at constant rate of oxygen consumption per gram of muscle. The combination of reduced ROS production, along with higher mitochondrial ROS protective enzymes (61), supports the idea that an additional benefit of chronic contractile activity, other than increased oxidative capacity, is an attenuated potential for ROS-mediated protein, lipid, or DNA damage (159). Conversely, the lower the mitochondrial content, as is evident in some tissues as a consequence of aging (118), the greater the tendency for ROS-induced damage to become evident, particularly in its effect on mtDNA. This highlights one potentially important reason for maintaining muscle mitochondrial content with a program of regular physical activity during the aging process.

Mitochondrial Biogenesis Requires the Cooperation of the Nuclear and Mitochondrial Genomes

One of the most fascinating aspects of mitochondrial synthesis is that it requires the cooperation of the nuclear and mitochondrial genomes (Fig. 2). Mitochondria are unique in the fact that they house multiple copies of a small circular DNA molecule (mtDNA) comprising 16,659 nucleotides. Although this mtDNA is minuscule compared with the 3 billion nucleotides found in the nuclear genome, it nonetheless contributes 13 mRNA, 22 tRNA, and 2 rRNA molecules that are essential for mitochondrial function. The thirteen mRNA molecules all encode protein components of the respiratory chain, responsible for electron transport and ATP synthesis.
Where does the cooperation between the genomes come in? First, these thirteen components comprise only a small fraction of the total respiratory chain proteins. Some act as single protein subunits, but many are combined with nuclear-encoded proteins to form multisubunit holoenzymes, like COX or NADH dehydrogenase (Fig. 2). The function of these holoenzymes is clearly impaired if contributions from either genome is absent (74). Second, it is known that mtDNA transcription and replication require the import of nuclear gene products, which act as polymerases or transcription factors (see Expression of mtDNA, below). Thus a longstanding question has been related to how the two genomes are regulated, or coordinated, in response to a stimulus leading to mitochondrial biogenesis. Williams et al. (190, 192) were the first to show that chronic contractile activity led to increases in mRNA levels encoding both nuclear and mitochondrial gene products. Subsequently, this was demonstrated for subunit mRNAs belonging to the same COX holoenzyme (82). Because COX contains 10 nuclear-encoded and 3 mitochondrial-encoded subunits, this enzyme is a useful model for studying the interactions of the two genomes. The mRNA expression of these subunits is also coordinated across a variety of tissues possessing a wide range of mitochondrial contents (54, 78). In addition, some evidence for a coordinated regulation of the two genomes was found during the mitochondrial biogenesis induced by cardiac hypertrophy (182), as well as in human muscle when trained and untrained individuals were compared (137). Given the diverse promoter regions of nuclear genes encoding mitochondrial proteins (108, 124), as well as the sequences of the mtDNA promoters, it is not surprising that this coordination can be disrupted. Evidence for this has been presented in cases of thyroid hormone treatment (80, 183), suggesting that a coordination of gene expression responses leading to strict stoichiometric relationships is not absolutely necessary for the formation of a functional organelle.

The Mitochondrial Reticulum Exists in Distinct Cellular Regions, Where It Possesses Different Biochemical Features

Although all mitochondria serve a similar function in providing ATP for energy demands of the cell, electron microscopy has revealed regional differences in the subcellular location of muscle and heart cell mitochondria (83, 127, 130). Mitochondria that are clustered in proximity to the sarcolemma are termed subsarcolemmal (SS) mitochondria, and those embedded among the myofibrils are called intermyofibrillar (IMF) mitochondria (83). SS mitochondria constitute ~10–15% of the
total mitochondrial volume and almost invariably adapt more readily to variations in muscle use (See Ref. 83 for review) or disuse (37, 101). These observations led to investigations designed to reveal the potential physiological role for these “mitochondrial populations” in muscle cells. Biochemical investigations have shown that isolated IMF mitochondria contain higher enzyme activities, respiratory rates, and import rates of precursor proteins but lower levels of the phospholipid cardiolipin (27, 168). It is not established how these differences arise, possibly related to different ATP demands in each subcellular region. It is surmised that SS mitochondria contribute ATP primarily for membrane function and that required by peripheral nuclei and that IMF mitochondria supply ATP to contracting myofibrils. Regardless, it is evident that the presence of clusters of heterogeneous populations of mitochondria can have profound effects on diffusion gradients within cells (7). In this respect, it has been hypothesized that the preferential increase in SS mitochondria in response to training can beneficially enhance the ability of the cell to take up and metabolize lipids (84).

The results of these biochemical studies were derived from mitochondria obtained using cell fractionation and differential centrifugation techniques. Although there is little doubt that the isolated fractions are reasonably pure, it is also true that mitochondria in muscle cells do not exist as discrete “populations.” Strong evidence points to a structure that more closely resembles a continuous mitochondrial membrane system (i.e., a reticulum or network; Refs. 10, 96). The presence of a reticular structure that interacts with organelles such as the cytoskeleton or the endoplasmic reticulum is also apparent in other cell types (50). This type of structure might have considerable advantage for the transport and metabolism of lipid precursors or for the involvement of mitochondria in calcium homeostasis. However, a reticular structure does not preclude the possibility that regional differences in membrane protein composition exist, as mitochondria in different locations may adapt to reflect diverse metabolic demands in distinct cellular regions (96). Differences in membrane protein composition within a continuous membrane unit are found in terminal fractions of sarcoplasmic reticulum or in subsynaptic plasma membrane regions (27), and these are retained during subcellular fractionation. It is also likely that species differences exist with respect to the extensiveness of a mitochondrial reticulum. There is some evidence of a partial mitochondrial reticulum in human muscle possessing a relatively low mitochondrial volume (2–8%; Refs. 83, 111). As the mitochondrial content increases, so does the likelihood of a reticular structure as the possibility of mitochondrial fusion increases (13). Evidence for this has been provided in horse muscle fibers in which a reticulum becomes more prominent in fibers with the highest mitochondrial content (94) and in rat (97) and rabbit (151) muscle during mitochondrial biogenesis induced by contractile activity.

CELLULAR MECHANISMS OF MITOCHONDRIAL BIOGENESIS

The initiation of mitochondrial biogenesis in muscle cells begins with putative signals brought about by muscle contraction. The magnitude of the signal(s), up to a point, is undoubtedly related to the intensity and duration of the contractile effort. This signaling can potentially lead to 1) the activation or inhibition of transcription factors, which will affect the rate of transcription, 2) the activation or inhibition of mRNA stability factors, which mediate changes in the rate of mRNA degradation, 3) alterations in translational efficiency, 4) the posttranslational modification of proteins, 5) changes in the kinetics of mitochondrial protein import, and/or 6) alterations in the rate of folding or assembly of proteins into multisubunit complexes. Additionally, the signal(s) could be transmitted directly to the mitochondria to initiate the replication or transcription of mtDNA or have an effect on mtRNA translation or holoenzyme assembly.

Initial Signaling Events

At the onset of contractile activity, a number of rapid events occur that could form part of the initial signaling process leading to downstream protein and lipid synthesis. These include 1) changes in the conformation of voltage-sensitive proteins responding to sarcoplasmic action potentials, 2) activation of cell surface integrin molecules, which are established mechanotransducers, 3) ion fluxes (e.g., Ca\(^{2+}\)) within the contracting muscle cell, 4) the extent of cross-bridge cycling and tension development, and 5) ATP turnover and the attendant stimulation of metabolism. With respect to mitochondrial biogenesis, the discussion below will be restricted to those events for which evidence exists (i.e., calcium signaling and ATP turnover). Excellent reviews exist on aspects of integrin signaling (23) and electrically mediated signaling events (155) as they relate to muscle plasticity.

Calcium. On its release from the sarcoplasmic reticulum, calcium permits actin and myosin interaction in muscle cells. It is also well recognized as an important second messenger in a variety of cell types (35), including muscle (102, 148, 177). Increases in the concentration of cytosolic calcium levels can activate a number of kinases [e.g., Ca\(^{2+}\)/calmodulin kinase II, protein kinase C (PKC)] and phosphatases (e.g., calcineurin), which ultimately translocate their signals to the nucleus to alter the rate of gene transcription (Fig. 2). Because of the tremendous fluctuations in cytosolic calcium levels during contraction and relaxation in both skeletal and cardiac muscle, attention has turned to the role of calcium in mediating muscle adaptation. Most interesting recently was the discovery that artificially induced changes in muscle calcium levels using a calcium ionophore could reversibly alter myosin isoform expression toward that of a slow phenotype, accompanied by an increase in mitochondrial enzyme activity (102). Subsequently, it was shown that fiber-type specific differences in calcium levels attained
during normal motor unit recruitment leads to the differential activation of calcineurin. Once active, calcineurin dephosphorylates the transcription factor nuclear factor of activated T cells and activates myocyte enhancer factor-2, which then bind to specific regions in the upstream regions of genes that are ultimately expressed in slow- but not fast-twitch muscle types (24, 196). This appears to be an essential determinant of the fast- and slow-twitch fiber composition of a muscle, but the involvement of calcineurin in regulating the activity of genes involved in mitochondrial biogenesis has not yet been investigated. However, it is clear that other calcium-responsive events have an impact on mitochondrial activity and gene expression. First, increases in cytosolic calcium are known to be matched within the organelle and directly influence the rate of mitochondrial respiration (93). This occurs via the activation of dehydrogenases, which require calcium for full activity (116). The change in mitochondrial calcium causes increases in ATP levels within both the cytosol and the mitochondria, which persist long after the mitochondrial calcium elevation has declined (92). Second, calcium-mediated changes in inner membrane phosphorylation events have been reported (8). These occur within the physiologic range of calcium concentrations and appear to involve the phosphorylation of subunit c of the F_{0}F_{1}-ATPase. Although the significance of these two findings is not yet established with respect to mitochondrial biogenesis, they may form part of an intramitochondrial signaling cascade (see Downstream Phosphorylation Events below). Third, decreases in calcium concentration within the medium surrounding cultured myotubes result in parallel changes in mitochondrial enzyme activities (156). Fourth, calcium-mediated increases in the expression of cytochrome c, an important component of the electron transport chain, have been documented (52). With the use of the calcium ionophore A-23187 to artificially increase intracellular calcium levels in L6 muscle cells, time- and concentration-dependent increases in cytochrome c mRNA levels have been reported, which were matched by increases in transcriptional activation (52). This increase in transcription was mediated, in part, by the activation of calcium-sensitive PKC isoforms, and it was removed, also in part, by inhibition of a mitogen-activated protein kinase (MAP kinase) pathway. There was no apparent involvement of calcium/calmodulin kinase II or IV in mediating the response. These data implicate both PKC and MAP kinase signaling in cytochrome c expression in muscle. Finally, studies of cells with experimentally induced mtDNA deletion have supported the conclusion that some calcium signaling events in muscle are propagated as a direct result of energy supply-demand imbalances. In mtDNA deletion, the synthesis of ATP is reduced because of a defective respiratory chain resulting from inadequate levels of mitochondrial gene products (14). This leads to an increase in cytosolic calcium concentration, presumably because the energy-dependent processes of calcium extrusion and uptake are impaired. An upregulation of a number of proteins related to calcium release and responsiveness (e.g., NFATc, calcineurin, ryanodine receptor-1), as well as nuclear genes encoding mitochondrial proteins, like COX subunit Vb, was observed (14). The interpretations of these data are that imbalances between cellular ATP demand and mitochondrial ATP supply, leading to alterations in calcium 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. However, it also appears that an increase in calcium cannot, by itself, lead to an augmentation in overall mitochondrial biogenesis. Subsequent studies using A-23187 have shown that, although a number of nuclear genes encoding mitochondrial proteins (e.g., malate dehydrogenase (MDH), the β-subunit of the F_{0}F_{1}-ATPase) are increased along with cytochrome c, a number of others that would be critical to mitochondrial biogenesis are not. For example, mRNAs encoding COX subunits (IV, Vb, and VIc) are unchanged, whereas those encoding glutamate dehydrogenase and mitochondrially encoded COX subunits II and III are decreased (Freysneset D, Ircher I, Connor MK, Di Carlo M, and Hood DA, unpublished observations). Thus calcium likely forms only part of a broader series of positive and negative signals that mediate changes in the synthesis of mitochondrial components.

**ATP turnover.** For at least 25 years, it has been hypothesized that disturbances in energy metabolism, leading to ATP depletion, an alteration in energy charge, or a change in the phosphorylation potential could initiate a compensatory response, ultimately leading to an increase in mitochondrial content (140). However, moderate-intensity exercise training can lead to mitochondrial biogenesis in the absence of marked changes in cellular ATP levels. Thus, although conditions of ATP depletion are known to lead to a higher mitochondrial content, it is likely that an increased rate of ATP turnover (i.e., increased rates of ATP synthesis and degradation) is sufficient to provoke mitochondrial biogenesis. These ideas have received considerable experimental support. First, experiments conducted using the drug β-guanidinopropionic acid to deplete the high-energy phosphate compounds phosphocreatine and ATP illustrated an upregulation of mitochondrial enzymes (162) and cytochrome c mRNA (103) in muscle. Second, as a result of accelerated ATP turnover, exercise increases the level of AMP and decreases the level of phosphocreatine in muscle, resulting in an activation of α2-AMPK (Fig. 1; Ref. 194). This activation can be simulated by administration of the drug 5-aminoimidazole-4-carboxamide-1-β-β-riboside to animals. After 4 wk of treatment, an increase in some (e.g., citrate synthase, succinate dehydrogenase, cytochrome c) but not all (e.g., carnitine palmitoyltransferase (CPT), hydroxyacyl-CoA dehydrogenase) mitochondrial enzymes was observed (187). This suggests that AMPK activation may be partially
involved in signaling the mitochondrial adaptation to contractile activity. Third, the hypothesis has received support from recent studies of mitochondrial uncoupling. Uncoupling refers to the dissipation of the electrochemical gradient across the inner membrane, which diminishes ATP production by disabling the activity of the F_{1}F_{0}-ATPase. Subsequently, electron transport and oxygen consumption are accelerated because control exerted by ADP is lost, and ATP utilization exceeds ATP synthesis. In this respect, the condition is analogous to intense exercise. Uncoupling can be achieved by using specific drugs in vitro, or by the overexpression of uncoupling protein (UCP). Within 6 h of uncoupling, an induction of the transcription factor nuclear respiratory factor-1 (NRF-1) was observed (109). NRF-1 has been widely implicated in mitochondrial biogenesis (153). Subsequent to the induction of NRF-1, an increase in the expression of NRF-1 target genes, including \( \delta \)-aminolevulinic acid synthase (ALAs), the rate-limiting enzyme in the synthesis of heme, was noted (109). As the prosthetic group in all mitochondrial cytochromes, heme is a vital component of the respiratory chain. Thus it appears that the increase in mitochondrial respiration, or the deficit between cellular ATP demand and mitochondrial ATP supply, provides a stimulus for the sequential induction of a variety of genes involved in the biogenesis of the organelle.

In summary, strong evidence exists to support potentially interactive and complementary roles for both calcium signaling and disturbances in ATP turnover as important contributors to the initiation of events leading to mitochondrial biogenesis. Further work is needed to determine the role, if any, of cross-bridge cycling and/or plasma membrane electrical events leading to the upregulation of specific target genes involved in mitochondrial function.

Downstream Phosphorylation Events

Evidence is plentiful regarding the positive effect of exercise on the activation of a variety of kinases that could potentially be involved in the phosphorylation of transcription factors. The results indicate that contractile activity per se, rather than humoral or neurotrophic factors associated with whole body exercise, activates the phosphorylation of kinases. This is a necessary requirement for the kinase to be potentially involved in contractile activity-induced mitochondrial biogenesis. Among those kinases activated by aerobic contractile activity are included PKC (142), the extracellular signal-regulated kinase (ERK)-1 and ERK2 MAP kinases (58, 66), p38 kinase (58), the p90 ribosomal S6 kinase (6), c-Jun NH2-terminal kinase (58), AMPK (184), calcium-independent serum response factor kinase (49), Raf-1 (161), and MAP kinase-activated protein kinase 2 (MAPKAP kinase-2) and mitogen- and stress-activated protein kinase 1 (MSK1) (149). Many of these results have been replicated in both rodent and human models of contractile activity. These data indicate the vast potential of contractile activity to alter kinase signaling pathways and subsequently the DNA binding activity of transcription factors or, alternatively, the RNA binding of stability factors via covalent modification.

A novel series of phosphorylation events also occurs at the level of the mitochondria, the implications of which remain to be fully established. It has been known for many years that certain subunits of the multisubunit complexes pyruvate dehydrogenase and branched-chain ketoacid dehydrogenase are reversibly phosphorylated. This can change the activation of the entire complex. Recently, other mitochondrial proteins, with more remote functions, have been found to be phosphorylated. These include COX subunit IV (164) and the 18-kDa subunit of complex I. The mechanism involved appears to be via the newly established presence of a cAMP-dependent protein kinase A located in the inner mitochondrial membrane (131). Interestingly, phosphorylation of the 18-kDa subunit activates mitochondrial respiration via an increase in complex I activity (152), whereas phosphorylation of subunit IV reduces respiration by inhibiting COX activity (12). This inhibition can be reversed by calcium-mediated activation of a protein phosphatase. It is also worth noting that calcium cannot only activate phosphatases but also has been shown to modulate the phosphorylation of subunit c of the F_{0}F_{1}-ATPase (8). The dominant influence leading to stimulation or inhibition of respiration may be determined by the presence of A-kinase anchoring proteins (AKAPs), which define the precise localizations of cAMP-dependent protein kinase A in various organelles (88). These data illustrate that important intracellular signaling pathways involving both calcium and cAMP extend beyond the cytosol and nucleus and into the mitochondria, where they certainly affect mitochondrial function and enzyme activity. Their roles in mitochondrial biogenesis have yet to be determined.

Transcription of Nuclear Genes

In general, transcription is regulated by proteins called transcription factors, which bind to upstream regulatory regions of genes. A number of these have been implicated in mitochondrial biogenesis, and they include NRF-1 and NRF-2, peroxisome proliferator-activated receptors-\( \alpha \) and \( \gamma \) (PPAR-\( \alpha \) and PPAR-\( \gamma \); Ref. 107), Egr-1 (zif268), and c-Jun, c-Fos, and Sp1 (108, 124). Contractile activity has been shown to induce increases in the mRNA and/or protein levels of several of these, consistent with their roles in mediating phenotypic changes as a result of exercise (2, 34, 119, 123, 126, 139, 161, 199). Nuclear genes encoding mitochondrial proteins possess upstream responsive elements to which these transcription factors bind to induce transcriptional activation. However, the upstream promoter elements of these genes are remarkably variable in their composition (108, 124), and only a few have been extensively studied. These include cytochrome c (see below) and subunits of the COX complex. The
variability among promoter regions suggests that a coordinated upregulation of nuclear gene transcription in response to contractile activity would be difficult to achieve, unless the multiple transcription factors mentioned above were effective in uniformly upregulating the transcriptional activity of numerous genes. One element that appears to be common to many promoters is the Sp1 site. However, recent evidence suggests that Sp1 may represent a negative regulator of transcription in some but not all genes (201). Thus the expression of nuclear genes encoding mitochondrial proteins in response to contractile activity could not possibly be coordinately upregulated if Sp1 acted alone to produce the transcriptional effect. However, it should also be considered that a complete coordination among the responses of all nuclear gene products is not achieved at the protein level (69) nor is it necessarily required, particularly if some gene products are found in excess. For example, an upregulation of cytochrome c but not Cox subunit IV expression has been observed in response to thyroid hormone treatment (144) and serum stimulation (71) in some cell types. In the latter condition, this was accompanied by an increase in mitochondrial respiration. Thus a coordinated increase in these two nuclear-encoded proteins was not necessary to achieve an increase in physiological function, even though changes in mitochondrial phenotype is a likely consequence of this differential induction.

A coordinated increase in the transcription of nuclear and mitochondrial genes would be achieved if a universal activator of the relevant genes was identified. In this respect, NRF-1 sites exist in the promoters of many nuclear genes, including mitochondrial transcription factor A (Tfam), as well as ALAs, the rate-limiting enzyme of heme synthesis. However, NRF-1 sites are not universally found. For example, not all of the 10 nuclear-encoded subunits of COX have NRF-1 sites in their upstream regulatory regions (108). Thus it cannot be expected that NRF-1 could coordinate transcription of these genes to produce a functional COX holoenzyme with stoichiometric increases in the synthesis of each subunit. However, the recent discovery of a coactivator of PPAR-γ termed PGC-1 has revealed it to be a potential regulatory candidate (197). Overexpression of PGC-1 was shown to induce morphologically measurable indexes of mitochondrial content, as well as the mRNA expression of a number of nuclear genes in muscle cells, including NRF-1, NRF-2α, Tfam, UCP-2, nuclear- and mitochondrial-encoded Cox subunits, and cytochrome c. The increase in Tfam expression, mediated by transcriptional activation of the Tfam promoter, led to an increase in mtDNA. This effect was probably a result of PGC-1 coactivation of NRF-1 on the Tfam promoter. Further evidence provided by loss-of-function experiments suggested that NRF-1 was necessary for the effect of PGC-1 on mitochondrial biogenesis. Subsequent work by Kelly and co-workers (173) showed that PGC-1 was capable of coactivating a second protein, PPAR-α, a receptor known to regulate the expression of enzymes involved in fatty acid oxidation such as medium-chain acyl CoA dehydrogenase (MCAD) and CPT-1. Using cardiac cells isolated during the perinatal period, they showed coincident increases in the mRNA expression of PGC-1 with the expression of MCAD, CPT-1, and PPAR-α (107). Furthermore, forced overexpression of PGC-1 led to increased expression of nuclear and mitochondrial genes located in the matrix and respiratory chain. This was accompanied by increased mitochondrial number and higher rates of coupled cellular respiration (107). Thus PGC-1 has emerged as a critically important protein in determining mitochondrial biogenesis and function. It is interesting to note that one of its binding partners, PPAR-α, is increased in muscle subject to chronic contractile activity (34), whereas another, PPAR-γ2, is located in the mitochondrial matrix (21). Further work will be required to determine the role that PGC-1 plays in regulating contractile activity-induced mitochondrial biogenesis in muscle.

mRNA stability

Cellular mRNA levels are a product of the rate of production (i.e., gene transcription) and the rate of degradation (i.e., mRNA stability). The stability of mRNA is usually mediated by protein factors that interact at the 3’ end of the transcript to either stabilise or destabilise the mRNA. These are expressed in a tissue-specific fashion, leading to wide variations in the stability of mRNA across tissues. In general, the stability of nuclear transcripts encoding mitochondrial proteins appears to be least stable in liver and most stable in skeletal muscle (30). For example, ALAs mRNA has a very low half-life of 22 min in liver but has a half-life of ~14 h in skeletal muscle (30). Thus mRNA stabilization may have the greatest consequence for mitochondrial biogenesis in liver, since modest increases in stability can have a marked effect on mRNA levels in this tissue. In this respect, mRNA stabilization has been shown to play an important role in the expression of the β-subunit of the F1-ATPase in liver during neonatal stages (90). Whether changes in mRNA stability are of benefit for mitochondrial biogenesis during muscle cell development is not yet known.

In Hep G2 cells in culture, the half-life of nuclear transcripts encoding mitochondrial proteins has been reported to range from 10 to 28 h (25). Remarkably, inhibition of mitochondrial protein synthesis led to a widespread increase in nuclear-encoded mRNA stability averaging approximately sevenfold. No effect was observed on those transcripts that were mitochondrially encoded (25). This suggests that a reduction in mitochondrial protein synthesis, which disrupts the assembly of the respiratory chain, could lead to an enhanced activity of RNA binding proteins that protect nuclear transcripts from degradation and results in increased mRNA levels. Interestingly, this is also observed in cells with respiratory impairment brought about by experimentally induced mtDNA depletion, in which a marked upregulation of mRNAs derived from multiple nuclear genes with products localized to the mitochondria (110, 178) is observed. In addition, pa-
tients harboring extensive mtDNA mutations exhibit large, compensatory increases in nuclear-derived transcript levels (68). The common feature of these diverse conditions is probably the reduced energy status of the cell or possibly the increase in ROS, which are produced when the electron transport chain is inhibited (18). The data suggest that deleterious modifications in respiratory chain structure, leading to reduced function (i.e., decreased ATP supply) relative to normal, represent a signal for the upregulation of nuclear-encoded gene products. This upregulation, often measured as mRNA levels on Northern blots, may largely be a product of increased mRNA stability. The data lend further support to the idea that a metabolically derived signal related to ATP turnover is involved in mediating mitochondrial biogenesis (see ATP turnover, above). It is also worth noting that the identity of the RNA binding proteins that confer stability or instability have remained largely unknown in most tissues, and even less is understood about mRNA stability in skeletal muscle or the role of contractile activity in modifying this process.

Example of a Nuclear Gene Encoding a Mitochondrial Protein: Cytochrome c

Cytochrome c has been used for many years as a representative indicator of muscle mitochondrial content. Its expression is influenced by variations in muscle disuse (15, 122), as well as by exercise intensity and duration (39). Its concentration is developmentally regulated (165) and influenced by thyroid status (154, 165). Most helpful in understanding the expression of cytochrome c was the characterization of the rat promoter. Evans and Scarpulla (46) found that both upstream and intronic elements were necessary for maximal promoter activity. The most important of these elements binds the protein NRF-1, and, together with a second functionally independent site referred to as region I, they represent the major upstream sequences that determine cytochrome c transcription (47). Binding sites for Sp1, found within the first intron, also confer cytochrome c promoter activity (Fig. 3; Ref. 47), and sites representing cAMP response elements (CRE) are also present but are likely active only in a tissue-specific context (59).

To gain an understanding of the regulation of cytochrome c expression resulting from contractile activity, electrical stimulation of neonatal cardiac myocytes (198, 199) was employed. The increase in cytochrome c mRNA observed after 48 h of stimulation was preceded by increases in the mRNA levels of NRF-1 (1–12 h) and c-Fos and c-Jun (0.25–3 h). Increases in cytochrome c transcriptional activation were observed initially at ∼12 h of stimulation. The stimulation effect was markedly attenuated if the NRF-1 site or the CRE were mutated. Both basal transcription and the magnitude of the stimulation response were reduced if the Sp1 site was mutated (199). Subsequently, it was shown that c-Jun, rather than CRE binding protein (CREB), was the major protein binding to the CRE that conferred transcriptional activation during electrical stimulation (198). These data illustrate the importance of c-Jun, NRF-1, and Sp1 in the transcriptional activation of cytochrome c, at least in heart cells (Fig. 3). Using skeletal muscle C2C12 cells, we have reported that contractile activity increases cytochrome c transcription, and that this is accompanied by an increase in Sp1 protein level and DNA binding after 4 days of stimulation (29). Additional studies in vivo have also indicated that cytochrome c expression can be regu-

Fig. 3. Partial description of cytochrome c expression in muscle during contractile activity. The schematic is derived from data published on both cardiac and skeletal muscle cells. A putative signal (see text) stimulates the transcription of nuclear respiratory factor-1 (NRF-1) and/or specificity protein 1 (Sp1). The mRNAs derived from these genes are exported from the nucleus to the cytosol via nuclear pores. These mRNAs are translated into protein in the cytosol, which are then translocated back into the nucleus. Contractile activity is known to increase the expression and DNA binding of both NRF-1 and Sp1, thereby enhancing the transcription of the cytochrome c (cyto c) gene. Many other nuclear genes encoding mitochondrial proteins are responsive to transcriptional activation by NRF-1 and Sp1, suggesting that these transcription factors are very important for mitochondrial biogenesis. C, COOH terminus; N, NH2 terminus.
lated by mRNA stability and that this may be the initial event involved. Freysenet et al. (51) employed a direct plasmid DNA injection technique to introduce the cytochrome c promoter, along with a chloramphenicol acetyltransferase reporter construct, into tibialis anterior muscles of animals undergoing unilateral chronic electrical stimulation (3 h/day) for 1–7 days. With the use of this technique along with an in vitro mRNA decay assay, time-dependent changes in the transcriptional activity and mRNA stability could be discerned. The results indicated that the increase in cytochrome c mRNA observed was initially due to an induced increase in mRNA stability between 2 and 4 days and that this was followed by transcriptional activation at 5 days of chronic stimulation. It was suspected that changes in mRNA stability might be involved in the regulation of cytochrome c mRNA because Yan et al. (200) had previously shown that the 3’ end of cytochrome c mRNA bound less of a yet unidentified destabilizing protein as a result of 9–13 days of chronic stimulation. This suggested that contractile activity led to the induction of an inhibitory factor responsible for decreasing the interaction between a destabilizing protein and the cytochrome c mRNA, thereby permitting its upregulation. These data emphasize the importance of recognizing mRNA stability as an important contributor to changes in gene expression resulting from contractile activity in muscle.

Mitochondrial Protein Import

Notwithstanding the importance of the mitochondrial genome in contributing proteins to the mitochondrial respiratory chain, it is nevertheless true that most mitochondrial proteins are derived from nuclear DNA. Therefore, a mechanism must exist for targeting these proteins to specific mitochondrial compartments once they have been synthesized in the cytosol. Most proteins are fabricated as “precursor” proteins with a signal sequence, often either located at the NH2 terminus or as an internal sequence (Fig. 4). Although pathways of protein targeting to the outer membrane, inner membrane, matrix, or intermembrane space differ somewhat from each other (98), the most widely studied path is that of proteins destined for the matrix. In this case, the positively charged NH2-terminal signal sequence interacts with a cytosolic molecular chaperone that unfolds the precursor and directs it to the outer membrane import receptor complex, termed the translocase of the outer membrane (Tom complex). Cytosolic chaperones include 70-kDa heat shock protein (HSP70) and mitochondrial import stimulating factor (MSF). On interaction with the translocase of the outer membrane (Tom complex), it is correctly oriented by interacting with the inner membrane phospholipid cardiolipin (not shown) before being transferred to the translocase of the inner membrane (Tim complex). The matrix chaperone mtHSP70 pulls in the precursor, and the signal sequence is cleaved by the mitochondrial processing peptidase (MPP). Subsequently, the mature protein is refolded by matrix chaperonins HSP60 and Cpn10. ATP is required at multiple steps during the import process. The number within each import machinery component refers to its size in kDa.
factor (MSF). Precursor proteins can be directed to one of two subcomplexes within the Tom machinery. One of these, consisting of the Tom20 and Tom22 receptors, is the preferential route for HSP70-chaperoned precursors. On the other hand, proteins interacting with MSF are largely directed to the Tom70-Tom37 heterodimer (120). Precursors are then transferred from the Tom receptors to Tom40 and the small Tom proteins 5, 6, and 7, which form an aqueous channel through which the precursor protein passes (112). Proteins are then sorted to the outer membrane, to the inner membrane, or to the translocase of the inner membrane (Tim), another protein complex that allows movement of precursor proteins to either the matrix or the inner membrane. Those proteins involved in the translocation of the precursor to the matrix are Tim17, Tim23, and Tim44. Tim17 and Tim23 act as integral membrane proteins, spanning the mitochondrial inner membrane and having domains associated with both the matrix and intermembrane space (133). In a manner similar to the Tim receptor complexes, Tim17 and Tim23 bind the precursor protein, prevent any untimely folding that would inhibit the precursor from translocating into the matrix, and form an aqueous pore through which the precursor can travel. In contrast, Tim44 is a peripheral membrane protein that is secured to the inner face of the inner mitochondrial membrane. Tim44 anchors the matrix chaperone HSP70 (mtHSP70), which acts in a ratchetedlike manner to pull the precursor into the matrix (Fig. 4). Along with these proteins, the inner membrane phospholipid cardiolipin is imperative for protein translocation because it appears to orient the precursor into the correct position for interaction with the Tim44-mtHSP70 complex. The importance of this phospholipid has been shown by studies in which cardiolipin function has been blocked using the drug Adriamycin, resulting in an attenuation of the import of proteins destined for the matrix (33, 42).

Two other elements are required for correct import of precursor proteins into the matrix. These are 1) the presence of an inner membrane potential (ΔΨ, negative inside) across the inner membrane to help pull the positively charged presequence into the matrix and 2) the availability of ATP both in the cytosol and in the matrix (168). Uncoupling agents that dissipate ΔΨ reduce protein import, whereas ATP depletion prevents the unfolding of the precursor in the cytosol and/or the action of mtHSP70 in the matrix (55). Thus reductions in cellular ATP levels such as that produced by severe contractile activity or defects in ATP production as might be encountered in cells with mtDNA mutations could affect the rate of import into mitochondria.

After its arrival in the matrix, the NH₂-terminal signal sequence is cleaved by a mitochondrial processing peptidase (MPP) to form the mature protein. It is then refolded into its active conformation by a mitochondrial chaperonin system consisting in part of 60-kDa heat shock protein (HSP60) and 10-kDa chaperonin (Cpn10). The vast majority of work that defines the components of the protein import machinery, as well as their cellular function, has been done in Saccharomyces cerevisiae and Neurospora crassa. This is now being extended to mammalian cells. For example, the kinetics of matrix precursor protein that import into skeletal muscle SS and IMF mitochondrial fractions, the ATP and cardiolipin dependence of the process, and the relationship to mitochondrial respiration have all been recently defined (168). IMF mitochondria import precursor proteins more rapidly than SS mitochondria, and there is a direct relationship between the capacity for mitochondrial respiration (and thus ATP production) and the rate of protein import (168). It has also been shown that a number of protein import machinery components are induced in response to chronic contractile activity. These include the chaperones MSF, cytosolic HSP70 (cHSP70), mtHSP70, HSP60, Cpn10, as well as the import receptor Tom20 (125, 129, 166).

Coincident with these increases are contractile activity-induced increases in the rate of import into the matrix but not into the outer membrane (166). This differential effect on protein targeting to mitochondrial compartments provides an example of how contractile activity can result in an altered mitochondrial protein stoichiometry. The accelerated rate of protein import into the matrix can be reproduced in cardiac mitochondria obtained from animals treated with thyroid hormone (33). Thus the effect is not a unique response to contractile activity but appears to be common to stimuli that increase mitochondrial biogenesis.

To more easily define the role of specific components of the import pathway in determining the kinetics of import, measurement of import in intact cells can be employed. When C2C12 cells were incubated with [35S]methionine and the import of radiolabeled MDH into mitochondria was measured, a greater rate of import was found during the progress of mitochondrial biogenesis occurring coincident with muscle differentiation (63). As expected, thyroid hormone accelerated the rate of import and induced the expression of Tom20. To evaluate the role of Tom20 alone in mediating the accelerated import rate, forced overexpression of Tom20 in these cells using a mammalian expression construct was used. Parallel increases in the rate of import and the magnitude of overexpression were observed. Conversely, inhibition of Tom20 expression using specific antisense oligonucleotides led to equivalent decreases in MDH import (63). These data suggest that the import of matrix-destined proteins is controlled, at least in part, by the expression of Tom20.

The protein import pathway represents an example of intracellular trafficking that is important for organelle biogenesis, and it may, under some conditions, determine the increase in mitochondrial content as a result of chronic exercise. For this to be the case, it must be shown that it is inducible and that it operates at a rate that limits the overall pathway under some conditions (i.e., chronic exercise). If the import rate was slow enough to limit mitochondrial biogenesis, then a pool of precursor proteins in the cell cytosol would be...
measurable. In the absence of such a pool, the assumption is that newly synthesized precursor proteins are rapidly taken up by mitochondria, and the kinetics do not limit the synthesis of the organelle as a whole. This has yet to be rigorously tested in a cellular system in which any other fates of the precursor (i.e., cytosolic degradation) are blocked. It is possible that the import of proteins might become limiting under conditions of chronic contractile activity if upstream steps (i.e., transcription, translation) are accelerated such that a saturating abundance of precursor proteins are presented to the import machinery. In any event, the physiological value of the observed contractile activity-induced increases in mitochondrial protein import is that mitochondria are more sensitive to changes in precursor protein concentration, a situation that would be advantageous for mitochondrial biogenesis at any given upstream production rate of cytosolic precursor proteins.

Progress in the area of protein import will advance substantially as additional mammalian homologues of the import machinery are identified. Recently, the first disease that can solely be attributed to a mutation in a protein component of the import machinery has been identified. A mutation in deafness dystonia protein (DPP) results in a neurodegenerative disorder characterized by muscle dystonia, sensorineural deafness, and blindness. DPP has been shown to be a mitochondrial protein that closely resembles Tim8p, a protein of the intermembrane space involved in the import process (99). In addition, mutations in the import receptor Tom70 have been shown to produce mtDNA rearrangements in the fungus *Podospora anserina*, presumably because of defective import of a component involved in mtDNA maintenance (91). The recent cloning of Tom22 (150), as well as members of the Tim machinery (11, 89, 175), will be of help in elucidating the functional roles of individual import machinery components in the import process and the relevance of import in mitochondrial based diseases and in organelle biogenesis.

**Expression of mtDNA**

Mammalian mtDNA genes are unevenly distributed on both the light and heavy strands of the circular genome (Fig. 4; Ref. 176). mtDNA contributes proteins that are vital to the function of the mitochondrial respiratory chain. Deletions or mutations of mtDNA, leading to defective or absent gene products, result in impaired mitochondrial respiration and mitochondrial disease, of which a large number have now been documented (176). These are largely tissue-specific diseases, located in organs with a high energy demand, such as brain, heart, and muscle. As expected, the result of a mtDNA abnormality in skeletal muscle is exercise intolerance (4), fatigue, and exaggerated lactic acid production (146).

mtDNA can replicate independently of nuclear DNA, and it is present in high copy number (10^3–10^4 copies) in virtually all cells of the body (111). Its replication requires the presence of DNA polymerase-γ (Pol-γ), a single-stranded binding protein (SSB), which facilitates Pol-γ activity, Tfam, which initiates transcription and generates primers to permit DNA replication, and a mitochondrial RNA-processing endonuclease (RNase MRP), which cleaves the nascent transcript to form the primer for replication (26).

Using the chronic stimulation model to induce mitochondrial biogenesis, Williams (188) showed that mitochondrial transcript levels correlated well with variations in mtDNA, as well as with the proportion of triplex DNA structure in the D-loop region (5). A close relationship between mtDNA and mtRNA was also found when muscles from trained and untrained individuals were compared (137) and in patients with a significant mtDNA deletion (67). These data suggest that the replication of mtDNA into multiple copies, or its specific conformation within the controlling D-loop region, regulates the level of mitochondrial gene expression in skeletal muscle cells. However, studies in muscle and other tissues suggest that this is not a universal finding. A variety of conditions appear to exist in which the copy number of mtDNA is not matched with the change in the level of transcripts or with changes in oxidative capacity (163, 181). Arguments can be made that, because both replication and transcription appear to be controlled by the action of Tfam and because replication relies on the formation of an mRNA primer, transcription may be the more important process of the two (105, 181). Whether control via mtDNA copy number is specific to contractile activity-induced mitochondrial biogenesis or whether differences in the results can be explained by variations in experimental approaches or measurement devices remains to be determined. In any event, the increase in mtDNA copy number observed as a result of chronic contractile activity is accompanied by the augmented expression of SSB (158), the RNA subunit of RNase MRP (128), and Tfam (60), but not Pol-γ (158). These data suggest that Pol-γ is sufficiently abundant and nonlimiting in the transcription of mtDNA.

Tfam has been extensively studied as the most important mammalian transcription factor for mtDNA. The level of Tfam correlates well with mtDNA abundance, and its loss, either in patients with mitochondrial myopathies (105, 136) or produced by experimental disruption of the Tfam gene (106), results in partial or total depletion of mtDNA. Homozygous Tfam knockout animals die before embryonic day 10 and are characterized by abnormal mitochondria and abolished oxidative phosphorylation (106). Animals possessing heart and muscle-specific disruptions of Tfam exhibit modestly reduced levels of mtDNA and mtRNA but little change in the activities of respiratory chain enzymes in skeletal muscle, possibly because of a low mtRNA turnover in this tissue (30, 179). However, these animals displayed characteristics found in the mitochondrial disease Kearns-Sayre syndrome, evident by dilated cardiomyopathy, abnormal mitochondrial morphology and proliferation, and a progressive atrioventricular conduction block (179). These data provide evidence for the importance of Tfam in normal
cardiac function, perhaps because the heart possesses the highest amount of mitochondria of all tissues. Furthermore, they illustrate the benefit of developing animal models to improve our understanding of the function of specific proteins and their role in mitochondrially based diseases (104, 176).

In skeletal muscle, Tfam expression and function is modified by contractile activity. During imposed chronic stimulation (3 h/day) ranging from 1 to 7 days in duration, Gordon et al. (60) recently showed an initial increase in Tfam mRNA which took place by 4 days. This was followed by accelerated import into mitochondria (5 days), increased mitochondrial Tfam protein content, and concomitantly higher DNA binding (7 days). This latter result was accompanied by parallel increases in COX III mRNA and COX enzyme activity (60). These data suggest that Tfam expression is well correlated with alterations in mitochondrial transcriptional activation and oxidative capacity. Thus the cellular events that limit Tfam expression may also act to restrain mitochondrial biogenesis as a whole. Because the increase in Tfam mRNA was the first noticeable event, it is likely that transcription of the Tfam gene plays an important role. However, given the remarkably slow import rate of Tfam into mitochondria compared with other matrix proteins (60), it also seems reasonable that accelerations in import rate, coincident with augmented Tfam expression as a result of contractile activity, complement each other in facilitating the overall rate of mtDNA replication and transcription.

A second transcription factor is now established as important in regulating mtDNA. Apart from Tfam, a 43-kDa protein known as p43 has recently been described that binds both thyroid hormone (T₃) as well as DNA sequences in the mtDNA D-loop control region (22, 195). This is an important finding because it helps to explain the direct action of T₃ on mitochondrial transcription in in vitro experiments using isolated mitochondria (22, 45). p43 was also shown to markedly influence muscle differentiation by enhancing mitochondrial enzyme activity and, as a result, the expression of the myogenic regulatory factor myogenin (147). If mitochondrial biogenesis in response to contractile activity is truly independent of hormonal (i.e., T₃) influences (see above), then p43 should play no role in the adaptation observed, and the only relevant transcription factor is likely Tfam. Indeed, it would be of interest to determine whether p43 has any ligand-independent (i.e., T₃-independent) influences or whether it exerts an additive effect with Tfam in determining the expression of mtDNA in muscle cells.

**Mitochondrial Protein Synthesis**

In addition to a separate genome, mitochondria also contain their own exclusive translational machinery. After cleavage of the polycistronic transcripts to form individual RNA molecules, these are used along with the rRNAs and tRNAs in the translation process. If one of the 22 tRNA genes is mutated, defective protein synthesis can result, causing severe clinical symptoms mainly in children and young adults, including hypertrophic cardiomyopathy and mitochondrial myopathy. Over 30 mtDNA protein synthesis mutations have now been described (176). Unfortunately, with the exception of a few studies in skeletal muscle (e.g., Refs. 144 and 145) and in heart (e.g., Refs. 32 and 117), very little information is available on the physiological regulation of mitochondrial protein synthesis in these tissues. In skeletal muscle, measurements in vitro revealed that isolated IMF mitochondria synthesize protein at a greater rate than SS mitochondria (27), consistent with the greater oxidative capacity found in the IMF subfraction. Recently, the effect of both acute and chronic contractile activity on protein turnover in these mitochondrial subfractions was described (28). Acute contractile activity (10 Hz stimulation, 5 min) reduced protein synthesis rates in SS mitochondria, which recovered to control levels with a 55-min recovery period or displayed continued contractile activity up to even 60 min (28). Protein degradation was not affected by 5 min of contractile activity but was reduced after 60 min of contractile activity followed by a recovery period. These data indicate that intramitochondrial protein synthesis and degradation rates are influenced by acute contractile activity in a time-dependent fashion. Certainly, the normal adaptive response of muscle to chronic contractile activity requires an intact mitochondrial translation system. Evidence for this was provided by experiments in which animals were treated with the mitochondrial protein synthesis inhibitor chloramphenicol during chronic stimulation-induced mitochondrial biogenesis. Reduced adaptive increases in the activities of COX but not citrate synthase (191) were observed. This is undoubtedly because COX contains three essential catalytic subunits derived from mtDNA that are translated within the organelle, whereas citrate synthase is entirely nuclear encoded. Given this requirement of protein synthesis for the normal muscle adaptation to exercise, it might be surmised that protein synthesis would be responsive to chronic contractile activity. Surprisingly, it was found that 14 days of electrical stimulation (3 h/day) of the rat tibialis anterior muscle led to reduced rates of protein synthesis in IMF mitochondria, whereas protein degradation was unaffected (28). These data support the possibility that any changes in protein turnover that occur as a result of exercise happen relatively early in the adaptation process, after which the process no longer appears to limit mitochondrial biogenesis in response to contractile activity.

**Recovery**

It has long been suspected that adaptive responses to chronic contractile activity are predominantly manifest during the recovery phase following the exercise period (17). With respect to mitochondrial biogenesis, Holloszy and Winder (77) showed that ALAs, the rate-limiting enzyme in heme metabolism, was increased.
several hours after the termination of the exercise bout. This was an important finding because this enzyme is involved in determining the functional content of mitochondrial cytochromes found in the respiratory chain. Similar results were observed in heart muscle (1) postexercise. To examine this further, Takahashi et al. (169) used chronic stimulation either acutely (one 3-h bout) or chronically (seven 3-h bouts), followed by 18 and 48 h of recovery and measured ALAs and COX activities along with ALAs mRNA levels. The results indicated that ALAs was increased during the recovery period but only after the 7-day stimulation period. Peak increases in ALAs activity occurred after 18 h, and they were matched by changes at the mRNA level. Interestingly, COX activity was also increased progressively during the recovery period in low-oxidative white muscle fibers (169). Subsequently, a number of studies have demonstrated increases in adaptive responses to various forms of contractile activity, which are evident during the recovery phase. These include increases in total muscle protein synthesis and degradation (134), increased activity of the 70-kDa S6 kinase (9), fiber-type-dependent changes in cAMP (160), increased mRNAs encoding GLUT-4, glycogenin, c-Fos, c-Jun, NRF-1, cytosolic HSP70, αB-crystallin, myoglobin, and citrate synthase (100, 123, 126, 138, 139), among others. As measured in human muscle, many of the increases in mRNAs encoding metabolic enzymes or proteins appear to be preceded by augmented rates of gene transcription (135). Together, these data appear to confirm that the final adaptations to various forms of contractile activity are a result of an accumulation of adaptive responses that originate with the first exercise bout but that are not manifest until sometime later, during the postexercise recovery period when the muscle is at rest. They also suggest that the recovery period is an important component of the adaptation phase of the genes necessary for the proliferation of mitochondria in muscle.

CONCLUSIONS AND FUTURE DIRECTIONS

Contractile activity initiates a series of physiological and biochemical events that lead to mitochondrial biogenesis. Those events that are now established are shown in Fig. 5, an illustration that is meant to briefly highlight recent work, to describe the approximate time course of the changes involved, and to provide a working model for expansion as our detailed knowledge improves. Note that the resulting alteration in muscle phenotype leads to a metabolic adaptation that reduces the “stress signal” imposed by an acute exercise bout of the same intensity and duration, acting in a negative feedback fashion. Thus the signal to produce further mitochondrial biogenesis is presumably reduced in magnitude once the adaptation has taken place.

Interest in mitochondrial function, biogenesis, and gene expression in muscle and nonmuscle cells has never been higher. This is because mitochondria provide an excellent adaptive model for the study of organelle biogenesis in mammalian cells and because mitochondria are now known to be implicated in a wide variety of diseases. Some avenues of future research that are likely to be rewarding include the following, enumerated below.

1) Understanding the relationship between the capacity for oxidative phosphorylation, the formation of ROS, its effects on mtDNA mutations, and its consequences can be studied. This work would have implications for improving our understanding of the decline in oxidative capacity in aging muscles. It would also permit us to forecast the benefit of regular exercise and the potential of maintaining oxidative capacity above a muscle-specific threshold, below which mtDNA damage and organ dysfunction ensue (i.e., mitochondrial myopathy; Ref. 176).

2) The role of chronic contractile activity in modifying the process of apoptosis can be evaluated. It is now fully established that mitochondria have an important role in triggering some forms of programmed cell death.
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