Mitochondrial Energetics and Therapeutics

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Abstract

Mitochondrial dysfunction has been linked to a wide range of degenerative and metabolic diseases, cancer, and aging. All these clinical manifestations arise from the central role of bioenergetics in cell biology. Although genetic therapies are maturing as the rules of bioenergetic genetics are clarified, metabolic therapies have been ineffectual. This failure results from our limited appreciation of the role of bioenergetics as the interface between the environment and the cell. A systems approach, which, ironically, was first successfully applied over 80 years ago with the introduction of the ketogenic diet, is required. Analysis of the many ways that a shift from carbohydrate glycolytic metabolism to fatty acid and ketone oxidative metabolism may modulate metabolism, signal transduction pathways, and the epigenome gives us an appreciation of the ketogenic diet and the potential for bioenergetic therapeutics.

Mitochondrial DNA (mtDNA): maternally inherited circular DNA located in the mitochondrial matrix; contains 13 critical oxidative phosphorylation subunits, 2 ribosomal RNA genes, and 22 transfer RNA genes

Mitochondrion: cytosolic organelle of endosymbiotic origin that produces energy by oxidizing substrates with oxygen by oxidative phosphorylation

nDNA: nuclear DNA

Reactive oxygen species (ROS): oxidative agents, generated as by-products of oxidative phosphorylation, that are central to the signal transduction systems of the cell but that, when produced in excess, can damage cellular mitochondrial, cytosolic, and nuclear components (DNA, proteins, and lipids)

Apoptosis: programmed cell death resulting in the activation of caspase enzymes, leading to cellular degradation. The intrinsic apoptosis pathway can be initiated via the mitochondrial permeability transition pore

mtPTP: mitochondrial permeability transition pore

INTRODUCTION

The role of energetic dysfunction in a broad range of metabolic and degenerative diseases, cancer, and aging is becoming increasingly apparent (1–3). Whereas the identification of mutations in mitochondrial DNA (mtDNA) genes 20 years ago (4–7) proved that energy deficiency could cause degenerative diseases, the pathophysiology of energy-deficiency diseases remains poorly understood. Consequently, although genetic therapies targeting energy-production genes have advanced in sophistication and potential effectiveness, metabolic therapies to ameliorate the symptoms of affected individuals have been slow to develop and disappointing in their effectiveness.

The disappointing progress in developing energetic medicines is the direct result of our very limited understanding of energy biology and the interrelationship between bioenergetics and environmental influences. Therefore, to advance energetic therapeutics, we must place mitochondrial diseases and their therapeutics in the broader context of organismal and cellular bioenergetics. This endeavor should not be confined to a few rare genetic diseases, as it may prove to be central to our understanding and treatment of a broad range of common metabolic and degenerative clinical problems.

At present, our knowledge of energetic diseases stems from the past 20 years of studies on mitochondrial bioenergetics and diseases. Mitochondrial diseases are heterogeneous and often multisystemic. Because the mitochondrion provides much of the energy for the cell, mitochondrial disorders preferentially affect tissues with high-energy demands such as the brain, muscle, heart, and endocrine system, although any organ can be affected. Thus, energetic defects have been implicated in forms of blindness, deafness, movement disorders, dementias, cardiomyopathy, myopathy, renal dysfunction, and aging. Moreover, because the mitochondria lie at the interface between environmental calories and organ energetic demands, the mitochondrion is the likely mediator of the common metabolic disorders. As a consequence,

mitochondrial dysfunction is probably central to diabetes, obesity, cardiovascular disease, and cancer.

Mitochondrial diseases can be caused by genetic defects in any mtDNA or nuclear DNA (nDNA) gene that encodes a mitochondrial protein or structural RNAs. Although mitochondrial diseases had been assumed to be rare, epidemiological studies have concluded that the frequency of mtDNA diseases alone is on the order of 1 in 5000 (8, 9), and known pathogenic mtDNA mutations have been detected in the cord blood of 1 in 200 live births (10). Furthermore, nDNA mutations affecting mitochondrial genes may be even more common than mtDNA mutations (3). Thus, the genetic burden of energy dysfunction is likely to be enormous. Because the mitochondria are believed to generate the majority of our energy, understanding the pathophysiology of rare and common mitochondrial diseases and the development of energetic therapies must start with the mitochondrion.

MITOCHONDRIAL BIOENERGETICS AND GENETICS

The mitochondria perform four central functions in the cell that are relevant to the pathophysiology of disease: They (*a*) provide the majority of the cellular energy in the form of ATP, (*b*) generate and regulate reactive oxygen species (ROS), (*c*) buffer cytosolic calcium (Ca^{2+}), and (*d*) regulate apoptosis through the mitochondrial permeability transition pore (mtPTP) (1).

Mitochondrial Energetics

Energetics in animals is based on the availability of reducing equivalents (hydrogen), consumed as carbohydrates and fats, that react with oxygen to generate water via mitochondrial oxidative phosphorylation (OXPHOS) (**Figure 1**). Glucose is cleaved into pyruvate via glycolysis within the cytosol, reducing cytosolic NAD^+ to NADH (reduced nicotinamide

adenine nucleotide). The pyruvate then enters the mitochondrion via pyruvate dehydrogenase (PDH), resulting in mitochondrial acetyl-CoA, NADH + H^+, and CO_2. The acetyl-CoA then enters the tricarboxylic acid (TCA) cycle, which strips the hydrogens from the organic acids, thereby generating NADH + H^+. Fatty acids are oxidized entirely within the mitochondrion by beta oxidation, generating mitochondrial acetyl-CoA, NADH + H^+, and $FADH_2$; the latter is contained in the electron transfer factor. Two electrons are transferred from NADH + H^+ to NADH dehydrogenase (complex I) or from $FADH_2$-containing enzymes such as the electron transfer factor dehydrogenase or succinate dehydrogenase (complex II) to reduce ubiquinone [coenzyme Q_{10} (CoQ)] to ubisemiquinone, and then to ubiquinol. The electrons from ubiquinol are then transferred down the remainder of the electron transport chain (ETC) successively to complex III (bc_1 complex), cytochrome c, complex IV [cytochrome c oxidase (COX)], and finally to oxygen ($\frac{1}{2}O_2$) to yield H_2O.

The energy that is released as the electrons flow down the ETC is used to pump protons out across the mitochondrial inner membrane through complexes I, III, and IV. This creates a proton electrochemical gradient ($\Delta P = \Delta \Psi + \Delta \mu^{H+}$), a capacitor that is acidic and positive in the intermembrane space and negative and alkaline on the matrix side. The potential energy stored in ΔP is used for multiple purposes: (*a*) to import proteins and Ca^{2+} into the mitochondrion, (*b*) to generate heat, and (*c*) to synthesize ATP within the mitochondrial matrix. The energy to convert ADP + Pi to ATP comes from the flow of protons through the ATP synthetase (complex V) back into the matrix. Matrix ATP is then exchanged for cytosolic ADP by the inner-membrane adenine nucleotide translocators (ANTs) (**Figure 1**) (11).

The efficiency with which dietary reducing equivalents are converted to ATP by OXPHOS is known as the coupling efficiency. This is determined by the efficiency with which protons

are pumped out of the matrix by complexes I, III, and IV and by the efficiency with which proton flux through complex V is converted to ATP. The uncoupler drug 2,4-dinitrophenol and the nDNA-encoded uncoupler proteins 1, 2, and 3 render the mitochondrial inner membrane "leaky" for protons. This short-circuits the mitochondrial inner-membrane capacitor; uncouples electron transport from ATP synthesis; and causes the ETC to run at its maximum rate, thereby dissipating the energy as heat.

Variation in mitochondrial proteins has been proposed to alter the OXPHOS coupling efficiency, thus altering the proportion of the calories burned by the mitochondrion that are allocated to ATP generation versus heat production. Alterations in the coupling efficiency can influence ROS generation, modulating Ca^{2+} uptake, and predilection to apoptosis. Such physiological changes permitted our ancestors to adapt to a range of new environments (12–15).

OXPHOS Complexes

The mitochondrion is assembled from genes encoded in both the nDNA and the mtDNA. OXPHOS complex I is assembled from 45 polypeptides, of which seven (ND1, -2, -3, -4, -4L, -5, and -6) are encoded by the mtDNA; complex II from four nDNA polypeptides; complex III from 11 polypeptides, of which one (cytochrome *b*) is encoded by the mtDNA; complex IV from 13 polypeptides, of which three (COI, -II, and -III) are from the mtDNA; and complex V from approximately 16 polypeptides, of which two (ATP6 and -8) are from the mtDNA. Of the five complexes, only complexes I, III, IV, and V transport protons, and they also retain mtDNA-encoded polypeptides. Because all of the proton-transporting complexes share a common electrochemical gradient (ΔP) that is central to the energetics of the cell, the proton transport of all four complexes must be balanced to avoid one of the complexes short-circuiting the common capacitor and negating the energy-generating capacity of the other

Oxidative phosphorylation (OXPHOS): process by which the mitochondrion oxidizes reduced substrates with oxygen to generate energy in a variety of forms, including ATP, heat, membrane potentials, and redox reactions

NADH: reduced nicotinamide adenine nucleotide

CoQ: coenzyme Q_{10}

complexes. Consequently, the major electrical components of the four proton-pumping complexes must coevolve. This is accomplished through the maintenance of all these proteins on a single piece of DNA inherited from only one parent, the mother in most animals. As a result, protein genes from different mtDNA maternal lineages with different coupling efficiencies cannot be mixed by recombination. Hence, all 13 mtDNA polypeptide genes are selected as a single unit, and only combinations of variants that optimally manage and utilize
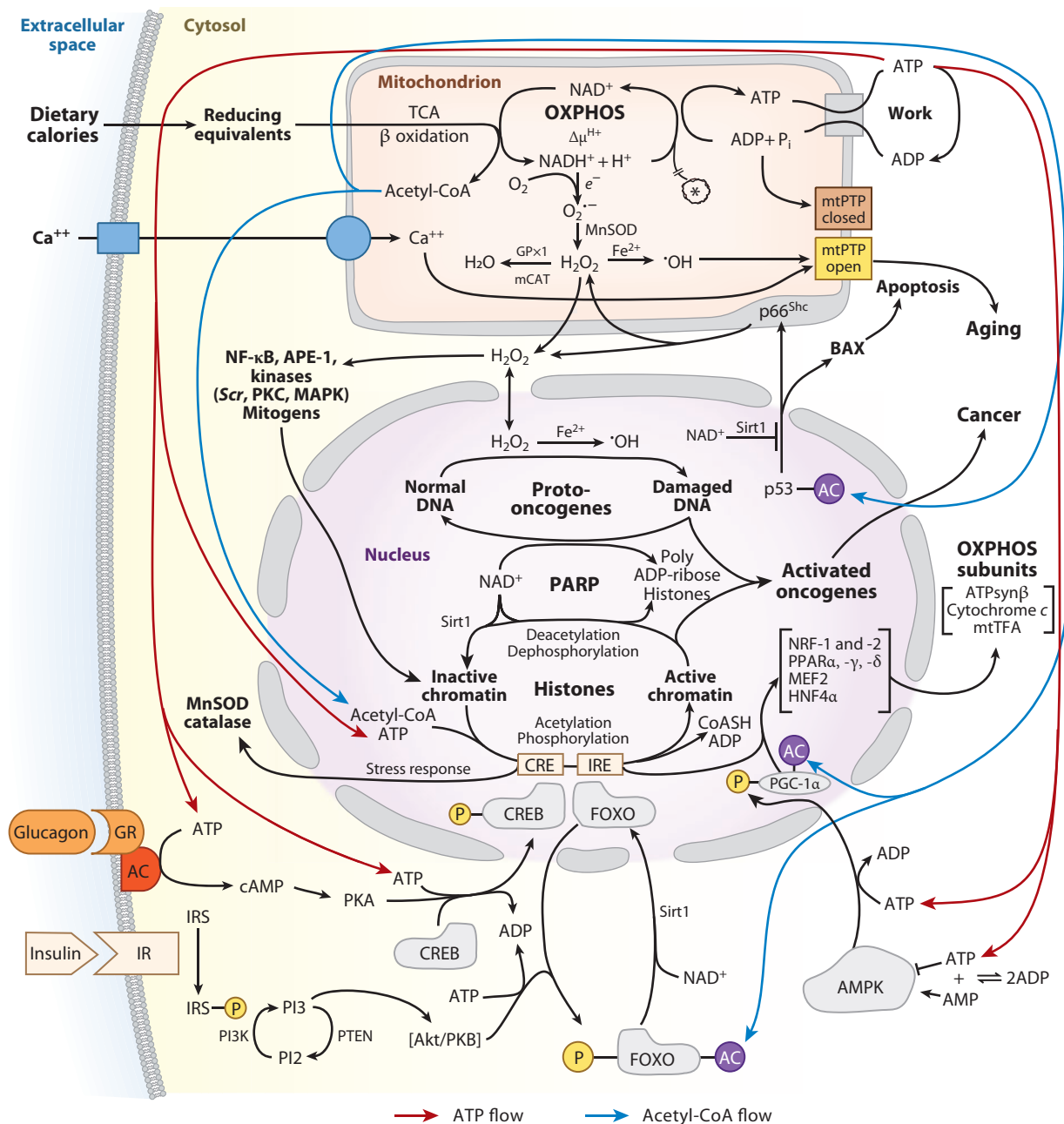
ΔP relative to environmental energy supplies and demands survive (11).

Each of the ETC complexes incorporates multiple electron carriers. Complexes I and II utilize flavins and iron-sulfur (Fe-S) centers. The TCA enzyme aconitase also utilizes an Fe-S group, which renders it sensitive to oxidative stress. Complex III encompasses an Fe-S center plus cytochromes b and c_1. Complex IV encompasses two Cu centers plus cytochromes a and a_3.

Mitochondrial Genetics

Although animal mtDNA encodes 13 core OXPHOS polypeptides plus the 22 transfer RNA (tRNA) and 12S and 16S ribosomal RNA (rRNA) genes for mitochondrial protein synthesis, the nDNA encodes the remaining 80% of the OXPHOS protein genes. In addition, the nDNA encodes all of the genes for the mitochondrial metabolic pathways and all of the enzymes required for mitochondrial

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Figure 1

Bioenergetic regulation of cellular physiology and gene expression. Dietary calories enter the cell as reducing equivalents. Carbohydrates are processed through glycolysis to generate cytosolic pyruvate and NADH (reduced nicotinamide adenine nucleotide). The pyruvate then enters the mitochondrion, is processed through pyruvate dehydrogenase, and is converted to acetyl-CoA, CO_2, and NADH. Fatty acids and ketone bodies enter the mitochondrion directly, where they generate acetyl-CoA and mitochondrial NADH. NADH can be oxidized within the mitochondrion by the electron transport chain to generate an inner-membrane electrochemical gradient ($\Delta P = \Delta\psi + \Delta\mu^{H+}$). This ΔP can then be used to generate ATP by the ATP synthase. The ATP is exported to the cytosol by the adenine nucleotide translocator (ANT) to energize work. Excess mitochondrial reducing equivalents can be transferred to O_2 to generate superoxide anion ($O_2 \cdot^-$). $O_2 \cdot^-$ is converted to hydrogen peroxide (H_2O_2) by manganese superoxide dismutase (MnSOD). H_2O_2 can diffuse out of the mitochondrion into the cytosol and nucleus. Further reduction of H_2O_2 results in hydroxyl radical ($\cdot$OH). The mitochondrial permeability transition pore (mtPTP) senses mitochondrial energy decline, reactive oxygen species (ROS) production, altered oxidation-reduction (redox) state, and increased Ca^{2+}. When activated, it opens a channel in the inner membrane, collapses ΔP, and induces apoptosis. Carbohydrate calories, in the form of glucose, are monitored by the pancreatic islet cells. High serum glucose elicits the secretion of insulin, which binds to the insulin receptors of target cells. This activates the phosphatidylinositol 3 kinase (PI3K) pathway to activate Akt protein kinase B (PKB). Akt phosphorylates the forkhead box, subgroup O (FOXO) transcription factors, barring them from the nucleus and their binding to insulin response elements (IREs). IREs are upstream of the mitochondrial transcription factor coactivator, peroxisome proliferator–activated receptor gamma coactivator 1 alpha (PGC-1α). In the absence of FOXO binding to IRE, PGC-1α transcription is reduced and mitochondrial biogenesis and oxidative phosphorylation (OXPHOS) decline, shifting metabolism toward glycolysis. When carbohydrates are limiting, serum glucose declines; insulin secretion diminishes; and the FOXOs become dephosphorylated and enter the nucleus, where they induce PGC-1α, upregulating mitochondrial biogenesis and OXPHOS. Furthermore, low glucose activates the pancreatic alpha cells to secrete glucagon. Glucagon binds to glucocorticoid receptors (GR) on target cells, activating adenylate cyclase (AC). cAMP activates protein kinase A (PKA) to phosphorylate cAMP response element binding (CREB), and phospho-CREB enters the nucleus, where it binds to cAMP response elements (CREs). One CRE is upstream of PGC-1α, resulting in its increased expression and the induction of mitochondrial biogenesis. Mitochondrial energetics (ATP and acetyl-CoA), redox status, and ROS also regulate cytosolic signal transduction pathways and the epigenome. Mitochondrial acetyl-CoA generated from pyruvate or fatty acids and ketones is converted to citrate. The citrate either drives the tricarboxylic acid (TCA) cycle to generate ATP or is exported to the cytosol and cleaved back to acetyl-CoA. Elevated cytosolic ATP and acetyl-CoA produced when calories are abundant can stimulate the phosphorylation and acetylation of histones, opening chromatin and stimulating transcription, growth, and cell replication. Diminished calories have the opposite effect. High acetyl-CoA also drives the acetylation and inactivation of the FOXOs and PGC-1α, shifting cellular metabolism away from OXPHOS and toward glycolysis. Glycolysis also causes the reduction of NAD^+ to NADH, but oxidation of fatty acids and ketones reduces mitochondrial NAD^+ to NADH but not cytosolic NAD^+. The cytosolic and nuclear protein deacetylase, Sirt1, requires NAD^+ as a coreactant and cannot use NADH. Therefore, during active glycolysis Sirt1 is inhibited, the FOXOs and PGC-1α remain acetylated, and the cell is biased toward glycolysis. However, during fatty acid and ketone oxidation, cytosolic NAD^+ remains oxidized, the Sirt1 deacetylates the FOXOs and PGC-1α, and OXPHOS is induced. Mitochondrial H_2O_2 is also an important agent in signal transduction, activating an array of kinases and other signaling molecules. However, excessive H_2O_2 production can damage cells and can act as a mutagen, potentially activating nuclear DNA (nDNA) oncogenes or inactivating tumor-suppressor genes. Abbreviations: AMPK, AMP-activated protein kinase; APE-1, apurinic/apyrimidinic endonuclease factor 1; HNF4α, hepatocyte nuclear factor 4 alpha; IR, insulin receptor; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; mCAT, mitochondrially targeted catalase; MEF2, myocyte-enhancing factor 2; mtTFA, mitochondrial transcription factor A; NF-κB, nuclear factor kappa B; NRF, nuclear regulatory factor; PTEN, phosphatase and tensin homolog.

Heteroplasmy:
situation in which two
different mtDNAs,
mutant and normal,
coexist in a cell

Homoplasmy:
situation in which the
mtDNAs in a cell are
either 100% normal or
100% mutant

biogenesis, including mtDNA polymerase gamma (POLG), RNA polymerase, mtDNA transcription factors, ribosomal proteins, etc. Therefore, the mitochondrial genome encompasses approximately 1500 nDNA-encoded mitochondrial genes plus 37 mtDNA genes (1, 3, 11, 16).

It is because the mitochondrial genome is dispersed between the uniparentally inherited mtDNA and the biparentally inherited nDNA that the genetics of energy metabolism is so complex. The mtDNA is a double-stranded, closed-circular molecule of 16,569 nucleotide pairs. In mammals, the two strands differ in their distribution of Gs and Cs, resulting in a C-rich light (L) strand and a G-rich heavy (H) strand. The gene arrangement of all mammals is conserved, and in addition to the structural genes the mtDNA contains a control region that encompasses the promoters for both H- and L-strand transcription plus the origin of H-strand replication. The origin of L-strand replication is located two-thirds of the way around the mtDNA in a clockwise direction. All of the rRNA and polypeptide genes are located on the H-strand, except for the ND6 gene, which is located on the L-strand. The tRNAs punctuate the genes, and the mature transcripts are generated from the polycistronic transcripts by cleavage of the tRNAs from the transcript, followed by polyadenylation of the mRNAs and rRNAs (17–20). The mtDNA genes have a much higher mutation rate than do the nDNA genes (21, 22). One likely reason for the high mtDNA mutation rate is the proximity of mtDNA to mitochondrial ROS production.

Each mammalian cell contains hundreds of mitochondria and thousands of mtDNAs. When a mutation arises in a mtDNA, it creates a mixed population of normal and mutant mtDNAs, resulting in a state known as heteroplasmy. When a heteroplasmic cell divides, the two types of mtDNAs are randomly distributed into the daughter cells, resulting in genetic drift toward either pure mutant or wild type. Over time, this replicative segregation results in segregation of the mutant mtDNAs into pure mu-

tant or normal populations, resulting in a state termed homoplasmy (11). As the percentage of mutant mtDNAs increases, mitochondrial energetic function declines. When energy output is insufficient for normal tissue function, a threshold is crossed, symptoms appear, and apoptosis or necrosis may be initiated (3, 23).

Human mtDNAs are strictly maternally inherited (24). Only one human exception—in an individual who developed mitochondrial myopathy—has been reported. His muscle tissue was found to have some paternal mtDNA containing a deleterious mutation (25).

MITOCHONDRIAL GENETIC DISEASES

A broad spectrum of complex clinical phenotypes has been linked to mutations in nDNA and mtDNA mitochondrial genes. Very different gene mutations can cause a similar range of phenotypes, mutations in the same gene can give a range of different phenotypes, and the same mtDNA mutation at different levels of heteroplasmy can result in totally different phenotypes. Some mtDNA mutations, such as those causing Leber hereditary optic neuropathy (LHON) (5), yield stereotypic phenotypes, whereas other mtDNA mutations, such as the tRNA mutations causing myoclonic epilepsy and ragged red fiber (MERRF) and the mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) syndromes, can result in highly variable multisystem diseases (3, 6, 7).

Mitochondrial DNA Diseases

Clinically relevant mtDNA variants fall into three classes: (*a*) recent deleterious mutations that result in maternally transmitted disease, (*b*) ancient adaptive variants that predispose individuals to disease in different environments, and (*c*) age-related accumulation of somatic mtDNA mutations that erode function and provide the aging clock (1). Pathogenic mtDNA mutations include both rearrangement mutations and base-substitution mutations.

Rearrangement mutations can be either de novo deletion mutations or maternally transmitted insertion mutations that are unstable and generate deletion mutations in postmitotic cells. Most deletion mutations remove at least one tRNA and thus affect protein synthesis (20). mtDNA-rearrangement syndromes are invariably heteroplasmic and can result in a range of clinical manifestations and severities. The mildest mtDNA-rearrangement phenotype is maternally inherited type 2 diabetes and deafness, thought to be caused by inheritance of an mtDNA-duplication mutation. The next most severe group of mtDNA rearrangement diseases comprises chronic progressive external ophthalmoplegia (CPEO) and the Kearns-Sayre syndrome (KSS), associated with ophthalmoplegia, ptosis, and mitochondrial myopathy with ragged red fibers (RRFs). RRFs are often associated with COX-negative and succinate dehydrogenase–hyperactive fiber zones in skeletal muscle that contain high concentrations of the rearranged mtDNAs (1, 20, 26, 27). The most severe mtDNA-rearrangement disease is the Pearson marrow/pancreas syndrome. Affected patients develop pancytopenia early in life and become transfusion dependent (28). If they survive the pancytopenia, they progress to KSS (29). Differences in mtDNA-rearrangement phenotypes appear to stem from differences between insertions and deletions, the diversity of tissues that contain the rearrangement, and the percentage of mtDNAs harboring the rearrangement in each tissue (20, 27).

Base-substitution mutations can alter either polypeptide genes (polypeptide mutations) or rRNAs and tRNAs (protein-synthesis mutations). Pathogenic polypeptide mutations encompass a broad spectrum of multisystem diseases, including LHON (5), Leigh syndrome (30), and mitochondrial myopathy (31–33), etc. Mitochondrial tRNA and rRNA protein-synthesis mutations can result in multisystem diseases such as MERRF (6, 7), MELAS (34), encephalomyopathy, mitochondrial myopathy and exercise intolerance, CPEO and KSS, gastrointestinal syndrome, dystonia, dia-betes, deafness, cardiomyopathy, renal failure, Alzheimer disease, Parkinson disease, etc. (3).

Both somatic and germline mtDNA mutations have been reported in cancers including renal adenocarcinomas, colon cancer cells, head and neck tumors, astrocytic tumors, thyroid tumors, breast tumors, prostate tumors, etc. (35, 36). Mitochondrial ROS production appears to be an important component of carcinogenesis. Introduction of the pathogenic human mtDNA ATP6 8993T>G missense mutation into prostate cancer cells increased mitochondrial ROS production and prostate tumor growth (37), and the presence of the mtDNA ND6 13997G>A mutation increased the metastatic potential of mouse cancer cell lines in association with increased mitochondrial ROS (38).

mtDNA lineages descended from a single founder mtDNA have accumulated sequential mutations along radiating mtDNA lineages, giving rise to maternal lineages encompassing groups of related mtDNA haplotypes (haplogroups). Haplogroups have generally been found to be associated with specific indigenous populations located in specific geographic regions. The human mtDNA sequence is highly variable, and approximately one-quarter to one-third of mtDNA polypeptide and structural RNA-sequence variants found in the general population appear to be functionally important. These observations suggest that functional mtDNA variants that altered mitochondrial coupling efficiency permitted individuals with that mtDNA to adapt to and multiply within new energetic environments. Natural selection then enriches for that mtDNA and all of its descendants that carried the beneficial variant to create a haplogroup specific for that region (12–15). Adaptive mtDNA haplogroups still correlate with the geographic distribution of indigenous people today. Two-thirds to three-quarters of all African mtDNAs belong to macrohaplogroup L; all European mtDNAs belong to macrohaplogroup N, encompassing the European haplogroups H, I, J, Uk, T, U, V, W, and X. Asian mtDNAs belong to either macrohaplogroup M

Threshold expression: percentage of mutant mtDNAs and/or severity of a biochemical defect that is sufficient to cause a specific clinical phenotype

or macrohaplogroup N. Of all of the Asian haplogroups, only A, C, and D became enriched in northeastern Siberia and crossed into the Americas on the Bering land bridge to found the Native American populations. These Native American haplogroups were subsequently joined by haplogroups B and X (1, 39).

These ancient adaptive mtDNA haplogroups still influence individual predisposition to a wide spectrum of common diseases. The first evidence that mtDNA haplogroups could modify disease predisposition was the discovery that European haplogroup J increases penetrance of the milder LHON mutations (40–43). It was subsequently shown that haplogroup H correlates with reduced risk of age-related macular degeneration and that haplogroups J and U are associated with increased risk of macular degeneration as well as increased drusen levels and retinal pigment abnormalities (44, 45). Haplogroups H and H-nt 4336 are associated with increased risk, and haplogroups J and Uk with decreased risk, for developing Parkinson disease (46–48). The haplogroup H-nt 4336 sublineage is also associated with increased Alzheimer disease risk, and haplogroups U and T are associated with decreased risk in certain contexts (49–52). Haplogroup J has been correlated with longevity in Europeans (53–56), and D has been correlated with longevity in Asians (57, 58). Haplogroup J has been associated with increased risk of diabetes in certain European descent populations (59–61), whereas haplogroup N9a is protective of diabetes, metabolic syndrome, and myocardial infarction in Asians (62–64). Haplogroup U has been associated with increased serum immunoglobin E levels (65), and haplogroup H has been associated with protection against sepsis (66). Haplogroups J and U5a are associated with more rapid progression of acquired immune deficiency syndrome, whereas H3, Uk, and IWX retard this disease's progression (67). Haplogroup H is protective against lipodystrophy associated with highly active antiretroviral therapy, but haplogroup T may increase risk of lipodystrophy (68). Finally, various haplogroups have been correlated with altered risk

for particular cancers (35, 36, 69–72). Therefore, mtDNA functional variation modulates predisposition to a wide range of metabolic and degenerative diseases as well as to cancer and longevity.

Mitochondrial Diseases of Nuclear DNA Mutations

Mutations in nDNA-encoded OXPHOS genes have also been linked to a variety of multisystem disorders ranging from lethal childhood Leigh syndrome to predisposition to depression (**Table 1**). These include mutations in structural and assembly genes of the respiratory complexes (73, 74), mutations in mitochondrial biogenesis genes such as POLG (75, 76), mutations in nucleoside and nucleotide metabolism (77–82), and mutations in genes involved in mitochondrial fission and fusion (83, 84).

MITOCHONDRIAL GENETIC THERAPIES

As our understanding of mitochondrial genetics has improved, approaches for the genetic therapy of mitochondrial diseases have also advanced. The modification of nDNA-encoded mitochondrial genes has drawn heavily on prior developments in the gene therapy of other Mendelian disorders. However, new gene-therapy approaches are required for mtDNA diseases because mutant mtDNA can be present in thousands of copies per cell, with the phenotype depending on the percentage of mutant and normal mtDNAs and each tissue's threshold expression.

Gene Therapy of Nuclear DNA Mitochondrial Mutations

Because many chromosomal mutations are recessive, introduction of a single functional gene into the nDNA should restore the missing biochemical function. In the case of recessive *ANT1* cardiomyopathy, adenoassociated virus (AAV) has been used to transduce the

Table 1 Examples of nuclear DNA mutations that cause mitochondrial diseases[a]

Gene name	Function	Chromosome	Inheritance	Clinical phenotype	Reference(s)
NDUFS8	Complex I	11q13	AR	Leigh syndrome	73
SURF1	Complex IV assembly	9q34	AR	Leigh syndrome	74
C10ORF2 (PEOA3)	Twinkle helicase, mtDNA stability	10q24	AD	AD-PEO, SANDO syndrome	75
POLG (PEOA1)	Polymerase gamma mtDNA replication, mtDNA stability	15q25	AD-AR	Alpers syndrome, AD and AR-PEO, SANDO syndrome, SCAE	76
ANT1 (PEOA2)	Adenine nucleotide translocator isoform 1, mtDNA stability	4q35	AD-AR	PEO, multiple mtDNA deletions	77, 78
DGUOK	Deoxyguanosine kinase, mitochondrial dNTP pool maintenance	2p13	AR	Hepatocerebral mtDNA depletion syndrome	79
TK2	Thymidine kinase, mitochondrial dNTP pool maintenance	16q22	AR	Myopathic mtDNA depletion	80
SUCLA2	Succinate-CoA ligase	13q12	AR	Leigh-like, dystonia, mild methylmalonic aciduria, mtDNA depletion	81
ECGF1	Thymidine phosphorylase, mtDNA stability	22q13	AR	MNGIE, multiple mtDNA deletions, depletion	82
OPA1	Dynamin-related protein	3q28-q29	AD	AD–optic atrophy	83
MFN2	Mitofusin, mitochondrial fusion	1p36-p35	AD	Charcot-Marie-Tooth disease 2A2	84

[a]Abbreviations: AD, autosomal dominant; AR, autosomal recessive; dNTP, deoxyribonucleotide triphosphate; MNGIE, myoneurogastrointestinal encephalopathy; mtDNA, mitochondrial DNA; PEO, progressive external ophthalmoplegia; SANDO, sensory ataxic neuropathy, dysarthria, and ophthalmoparesis; SCAE, spinocerebellar ataxia with epilepsy.

heart-muscle isoform of *Ant1* into the skeletal muscle of mice in which the *Ant1* gene has been genetically inactivated. *Ant1*$^{-/-}$ mice develop mitochondrial myopathy with RRFs and proliferation of abnormal mitochondria, but without external ophthalmoplegia, together with progressive cardiomyopathy (85, 86). Exactly the same phenotype has been reported for a patient harboring a homozygous *ANT1* null mutation (77). The skeletal muscle of mice transduced with the AAV-*Ant1* virus showed a 25% increase in muscle enzyme and ATP levels as well as a strong reversal of the histological abnormalities associated with the disease (86). Therefore, gene transduction of nDNA-encoded mitochondrial genes should be a feasible strategy for recessive mutations.

Gene Therapy of Mitochondrial DNA Mutations

To address mtDNA diseases, we could influence the percentage of mutant and normal mtDNAs and/or change the structure and function of the mitochondrial gene. Therefore, investigators are addressing mtDNA mutations with three different approaches: (*a*) import of normal mtDNA polypeptides into the mitochondrion to complement the mtDNA defect, (*b*) reduction of the proportion of mutant mtDNAs (heteroplasmy shifting), and (*c*) direct medication of the mtDNA.

Mitochondrial import polypeptides: allotopic versus xenotopic. Of the more than 200 pathogenic mtDNA mutations that have

been published, approximately one-third are located in mtDNA-encoded polypeptide genes (3, 87). For treatment of polypeptide mutations, one approach under development is to isolate a normal copy of the mutant mtDNA polypeptide gene, adjust its genetic code to that of the nDNA, introduce appropriate transcriptional regulatory elements and an amino-terminal mitochondrial targeting peptide, and introduce the resulting allotopic gene into the nDNA. It is hoped that a sufficient amount of the allotopic protein would be introduced into the mitochondrion to overwhelm the high level of mtDNA mutant protein, partially correcting the biochemical defect and leading to suppression of the pathological symptoms.

Allotopic expression has already been attempted with some success in cultured cells. The human *ATP8* gene has been reengineered for allotopic expression and successfully introduced into the nucleus, and its protein has been transported back into the mitochondrion (88). The mtDNA *ATP6* gene has also been reengineered and introduced into the nucleus, and it has reportedly been able to biochemically rescue human transmitochondrial cybrids harboring the homoplasmic mtDNA 8993T>G mutation (89). Allotopic expression of the mtDNA *ND4* gene has also been reported to restore biochemical function to cybrids harboring the *ND4* 11778G>A missense mutation responsible for LHON. The aldehyde dehydrogenase–targeting peptide was used to direct the reengineered ND4 protein to the mitochondria, thereby partially restoring mitochondrial ATP production (90).

A large proportion of pathogenic mtDNA mutations alter the structure and function of mtDNA tRNAs. Although it is thought that all or virtually all of the mammalian mtDNA tRNAs are encoded by the mtDNA, in other organisms a portion of the mitochondrial tRNAs are encoded by nDNA genes and imported into the mitochondrion. Therefore, mtDNA tRNA mutations may be complemented by introduction of a xenotopic nDNA-encoded yeast mitochondrial tRNA

into the mammalian nucleus and having the yeast tRNA imported into the mitochondrion to complement the defect. This approach has been attempted for the treatment of human cells harboring the *tRNA^Lys* nucleotide 8344A>G mutation with the yeast *tRNA^Lys* nDNA gene. This partially restored the mitochondrial dysfunction associated with the mitochondrial protein-synthesis defect (91). Similarly, the *Leishmania* mitochondrial RNA import complex has been used to introduce, through a caveolin-1-dependent pathway, the human cytosolic *tRNA^Lys* into human cybrids harboring the *tRNA^Lys* 8344A>G mtDNA mutation. Significant restoration of mitochondrial function was observed (92).

The yeast *Saccharomyces cerevisiae NDI1* gene encodes a single polypeptide NADH oxidase, which performs the NADH-CoQ oxidation-reduction (redox) reaction without pumping protons. The introduction of *NDI1* into the nDNA of cell lines harboring LHON mtDNA complex I mutations through use of an AAV vector has been reported to restore electron transport activity, although the mitochondria lacked one proton-pumping (coupling) site. The AAV-*NDI1* vector was found to transduce mouse brain and skeletal muscle (93). AAV-*NDI1* transduction has also been shown to be neuroprotective when injected into the substantia nigra of mice treated with the complex I inhibitor, rotenone, which results in a Parkinson-like disease in rats. *NDI1* transduction reduced cell death and oxidative damage of the dopaminergic neurons (94).

AAV transduction has also been used to induce a disease similar to LHON. The retina and optic nerve were transduced with viruses carrying ribozymes that destroy the mRNAs for either mitochondrial manganese superoxide dismutase (MnSOD; *Sod2*) (95) or the nDNA-encoded *NDUFA1* subunit of complex I (96). The loss of optic nerve cells resulting from *NDUFA1* ribozyme–transduced cells could then be partially ameliorated by a secondary transduction of the retina with an AAV carrying the human *Sod2*. The AAV-*Sod2* transduction was found to reduce apoptosis in the retinal

ganglion cells and the accompanying degeneration of the optic nerve (97).

The allotopic expression of the cyanide-insensitive alternative oxidase (AOX) from the ascidian *Ciona intestinalis* has been used to confer significant cyanide-insensitive COX activity in cultured cells. Transduction of this enzyme into the nucleus both bypassed a patient's COX defects and prevented overreduction of the mitochondrial quinone pool responsible for ROS, thus acting as an antioxidant (98). Moreover, transforming mouse mtDNA-deficient (ρ°) cells with the *S. cerevisiae* NADH dehydrogenase gene (*Ndi1*) and the *Emericella nidulans Aox* gene restored NADH/CoQ plus CoQ oxidase activity, relieving the ρ° cell's dependency on exogenous pyruvate and uridine (99).

Heteroplasmic shifting. Because the threshold proportion of mtDNAs necessary to result in a clinically relevant biochemical defect can occur over a narrow range (generally a few percent), various approaches have been used to shift the proportion of mutant mtDNAs to below the clinical expression threshold. This heteroplasmic shifting strategy has been achieved in cultured cells containing a mixture of wild-type and deleted mtDNA molecules when the heteroplasmic cells were maintained in a ketogenic medium. This medium lacked glucose but contained either beta-hydroxybutyrate or acetoacetate, or both. This obligatory oxidative medium resulted in an increase in wild-type mtDNAs from 13% to 22% within 5 days (100). Similarly, in cybrid clones heteroplasmic for the *ATP6* 8993T>G mutation associated with a L156R amino acid substitution, growth in medium containing the ATP synthase inhibitor oligomycin to accentuate the ATP synthase defect and galactose to enhance the cellular dependency on mitochondrial OXPHOS caused the wild-type mtDNAs to increase from 16% to 22% (101).

A more direct approach has been to introduce into the nDNA of a heteroplasmic cell, a mitochondrially targeted restriction endonuclease that recognizes and cleaves the mtDNAs harboring the pathogenic mutation but spares the mtDNAs with the normal sequence. Through this approach, cells heteroplasmic for the *ATP6* 8993T>G mutation have been shifted toward the wild-type 8993T mtDNA via a mitochondrially targeted *Sma1* restriction endonuclease (102). A similar approach has been used in vivo to shift the mtDNA heteroplasmy level in muscle and brain of NZB/BALB heteroplasmic mice through the introduction of a transgene that targets the *Apa*LI restriction endonuclease to the mitochondria. The BALB mtDNA has an *Apa*LI restriction site that the NZB mtDNA lacks (103).

Direct modifications of the mitochondrial DNA. Attempts to directly correct mtDNA mutations through the introduction of exogenous DNA into the mitochondrion have not as yet been routinely successful. In an alternative approach, protein nucleic acids (PNAs) have been introduced into cellular mitochondria in an effort to inhibit the replication of the mutant mtDNAs. PNAs substitute a peptide backbone for the sugar phosphate background of nucleic acids. Because peptide bonds are not charged, the PNAs bind with a much higher affinity to complementary nucleic acid strands than to regular nucleic acid strands. PNAs have been synthesized to be complementary either to the *tRNA^{Lys}* nucleotide 8344A>G mutation or to the breakpoint junction associated with the common 4977 nucleotide deletion. The PNAs are taken up by cells and inhibit the replication of the mutant mtDNA between 75% and 80% without affecting the replication of the wild type. However, in vivo efficacy of this approach has yet to be established (104, 105).

The permeability of PNAs to mitochondria has also been used to introduce oligonucleotides into the mitochondrial matrix. Oligonucleotides complementary to the mtDNA have been annealed to complementary PNAs, which in turn were linked to synthetically synthesized N-terminal mitochondrial-targeting sequences. These conjugates were then introduced into cells by various procedures, including streptolysin permeabilization. Once the conjugate was inside the cytoplasm, it

was selectively imported into the mitochondrial matrix (106). Assuming that an oligonucleotide with the wild-type sequence can dissociate from the PNA and anneal to the single-stranded mtDNA replication intermediate, the oligonucleotide containing the wild-type base could act as a primer and replace the mutation in the replicating strand. However, this conjecture has not yet been shown to be feasible.

Stem Cell and Organ Transplantation Therapy of Mitochondrial Disorders

As an alternative to correcting a mutated nDNA or mtDNA mitochondrial gene, it may be possible to replace the cells of the affected mutant tissue with stem cells. Such replacement has been achieved for mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), an autosomal recessive progressive and fatal disorder resulting from mutations in the thymidine phosphorylase gene (*TYMP*). Absence of the *TYMP* gene results in the accumulation of toxic levels of thymidine and deoxyuridine in blood. The introduction of allogeneic stem cells, which produce the enzyme and release it into the blood stream, may then establish the enzyme-producing cells in various organs and eliminate the toxic serum metabolite. In one MNGIE patient, this approach resulted in the nearly complete reduction of the toxic blood substrates, as well as significant clinical improvement (107).

Organ transplantation is another approach for correcting specific mitochondrial disease pathologies. This approach has been successful in treating certain mitochondrial diseases, including cardiomyopathy (108) and liver failure (109).

Prenatal Diagnosis, Preimplantation Genetics, and Germline Gene Therapy

Although the above approaches show promise for most patients, women harboring a deleterious mtDNA mutation have a very substantial risk of bearing children with debilitating mito-

chondrial disease. Because the transmission of a pathogenic mtDNA mutation is stochastic, the mtDNA genotype of chorionic villi or amniocytes may not provide a reliable estimate of the mtDNA mutational load in the target tissues (110, 111). Although the precise genotype of a fetus for the MELAS 3243A>G mutation cannot be deduced, the demonstration that fetal cells have very low mutant mtDNA has been considered reassuring (112). By contrast, placental and amniotic mutation loads have been found to reflect fetal tissue heteroplasmy levels for the ATP6 8993T>G mutation, potentially enabling limited prenatal predictions for this mutation (113).

Preimplantation genetic diagnosis appears to be a promising new approach for the mtDNA-inheritance dilemma. The oocytes of the mtDNA mutation carrier (the mother) could be fertilized in vitro, and either a polar body or a blastomere could be collected and the mtDNA heteroplasmic genotype determined. The embryos with the lowest proportion of mutant mtDNAs could then be selected for implantation (114, 115). Adoption or oocyte donation provide other reproductive alternatives for families with maternally inherited mtDNA disease (116).

A more direct, albeit more complex, approach to eliminating maternally inherited pathogenic mutations may be germline mtDNA therapy. One proposal is to fertilize the mother's mutant oocytes in vitro, then remove the zygote or early blastomere nucleus by micropipette and inject it into an enucleated oocyte from a woman with normal mtDNA. Implantation of this nuclear transplantation embryo into the mother's uterus should result in child(ren) with the nDNA from a normal conception by the parents, but with nonmaternal and thus normal mtDNA. If the recipient oocyte were donated by the father's sister, the mtDNA lineage would simply switch from the maternal to the paternal side (114, 117). Because some of the mother's mitochondria might be transmitted along with the nucleus during the nuclear transplantation, creating an undesirable heteroplasmy, any residual maternal

mitochondria and mtDNAs might be eliminated by rhodamine 6G treatment (118).

MITOCHONDRIAL AND CELLULAR BIOENERGETICS

Although these genetic approaches could help ameliorate the symptoms of some patients with nDNA-encoded mitochondrial diseases and could assist with the reproductive decisions of women harboring deleterious heteroplasmic mtDNA mutations, they provide little hope of relieving the debilitating symptoms of most patients with mitochondrial disease. What is required is an effective metabolic or pharmacological treatment that ameliorates these symptoms. Although numerous case reports and a limited number of clinical trials on putative mitochondrial therapies have been conducted, marked reproducible clinical improvement has been difficult to verify.

In a recent review on treatment of mitochondrial disorders, the so-called Cochrane collaboration concluded that no clear benefit from the main components of the mitochondrial therapies could be scientifically established. The Cochrane collaboration judged only seven clinical trials to be scientifically valid. These trials encompassed a variety of compounds such as CoQ, creatine monohydrate, dichloroacetate, and dimethyglycine, but they used limited sample sizes of 5 to 30 patients (119, 120).

Beyond these controlled clinical trials, there is an extensive literature comprising open-label or retrospective studies and single case reports. However, these reports often present conflicting results. Thus, the development and testing of metabolic therapies have been plagued by the limited availability of groups of well-characterized patients with similar molecular defects and by our imperfect understanding of the pathophysiology of mitochondrial diseases.

Given that mitochondrial dysfunction involves a perturbation of metabolism, why have metabolic and pharmacological treatments not been more effective? We believe that the answer lies in the integrated network of interactions within the human cell, in which mitochondrial bioenergetics is involved. A single energetic defect can have many different consequences. Thus, use of a single metabolite such as ascorbate (vitamin C) or tocopherol (vitamin E) to perturb the system may have only a transient effect, given that the other components of the network ultimately counteract the therapeutic intervention. Furthermore, the varying energetic defects in different patients perturb the network in different ways. Hence, the same therapeutic approach may be beneficial for one individual and deleterious for another.

Therefore, to develop effective metabolic and pharmacological therapies, we must understand the energetics network. Then we could develop therapeutic strategies to address the various ramifications of each bioenergetic defect, thus permitting coordinated targeting of multiple different dysfunctional consequences of the genetic mutation.

Energetic Regulation

To understand mitochondrial bioenergetics, we must start with the source of our energy: the dietary calories that we consume. The environmental calories consumed determine the availability of reducing equivalents for the mitochondrial redox reactions (**Figure 1**).

In the presence of excess calories, cells are programmed to actively catabolize the available calories and use the energy for cellular growth, maintenance, repair, and—in the case of proliferative cells—to replicate their DNA and divide. We have hypothesized that the energetic status of the cell is communicated to the nucleus by the modification of nuclear chromatin through phosphorylation via ATP, through acetylation via acetyl-CoA, and through methylation via S-adenosylmethionine—all high-energy intermediates generated via glycolysis and mitochondrial metabolic and OXPHOS pathways. Thus, when calories are abundant, chromatin histones become phosphorylated by kinases and acetylated by histone acetylases, which reduce their affinity for DNA and permit active gene expression. In the absence of

sufficient calories, ATP and acetyl-CoA levels decline, histone phosphorylation and acetylation are reduced, gene expression is repressed, and growth declines and ultimately stops.

In multicellular organisms, this system has become modified such that when carbohydrates in the diet are abundant, cellular metabolism shifts toward glycolysis with the excess calories being stored as fat in the white and brown adipose tissues. When carbohydrate supply is limited, energy must be generated through oxidation of fatty acids by mitochondrial beta oxidation. The shift toward a more glycolytic metabolism is achieved because high serum glucose stimulates pancreatic beta cell insulin secretion, which acts on target cells through phosphatidylinositol 3 kinase (PI3K) and Akt to phosphorylate and inactivate the forkhead box, subgroup O (FOXO) transcription factors. Inactivation of the FOXOs suppresses the expression of the mitochondrial gene cotranscriptional activator, peroxisome proliferator–activated receptor (PPAR) gamma coactivator 1 alpha (*PGC-1α*), thus downregulating mitochondrial OXPHOS and antioxidant defenses. In the high-carbohydrate state, acetyl-CoA is abundant and glycolysis generates excess cytosolic NADH. This process results in the acetylation and partial inactivation of the FOXOs and PGC-1α, which are stable because deacetylation by the protein deacetylase Sirt1 requires NAD^+ as a substrate and Sirt1 cannot use NADH. When carbohydrates are limited, insulin secretion declines, the FOXOs become dephosphorylated, and PGC-1α transcription increases. Moreover, the pancreatic alpha cells secrete glucagon-activating adenylylcyclase in the target tissues. The resulting cAMP stimulates protein kinase A to phosphorylate the cAMP response element binding protein (CREB), which also increases the transcription of PGC-1α. Moreover, because oxidation of fatty acids and of the liver-generated ketone bodies (beta-hydroxybutyrate and acetoacetate) can only occur within the mitochondrion, the cytosolic NADH/NAD^+ ratio remains low. The high proportion of cytosolic NAD^+ then stimulates Sirt1 to deacetylate

the FOXOs and PCG-1α, thus enhancing mitochondrial biogenesis, OXPHOS activity, and the antioxidant enzyme level. These changes facilitate the extraction of the calories from stored fats and circulating ketones (**Figure 1**) (11, 121).

The induction of mitochondrial biogenesis can be further stimulated by fasting and starvation through the action of AMP-activated protein kinase (AMPK). When ATP is limited, the increased AMP activates AMPK, which phosphorylates and further activates PGC-1α to upregulate mitochondrial biogenesis.

As mitochondrial OXPHOS increases, hydrogen peroxide (H_2O_2) production also increases. The mitochondrial H_2O_2 can then diffuse out of the mitochondrion and into the nucleus, changing the nuclear redox potential and altering the redox status of transcription factors. In potentially mitotic cells, the mitochondrial H_2O_2 can activate immediate early transcription and induce the replication cycle (**Figure 1**). This mitogenic character of subtoxic levels of H_2O_2 has been well documented in cultured mammalian cells (122).

Reactive Oxygen Species and Reactive Nitrogen Species

The mitochondria generate much of the endogenous cellular ROS through OXPHOS. Under normal physiological conditions, ROS production is highly regulated, at least in part, by complex I (123–127). However, when the ETC is inhibited by an OXPHOS gene mutation or becomes highly reduced from excessive calorie consumption relative to exercise level, then the ETC electron carriers accumulate excessive electrons, which can be passed directly to O_2 to generate superoxide anion ($O_2 \cdot^-$). The $O_2 \cdot^-$ generated by complex I is released into the mitochondrial matrix, where it is converted to H_2O_2 by MnSOD (the *Sod2* gene). The $O_2 \cdot^-$ generated from complex III is released into the mitochondrial intermembrane space, where it is converted to H_2O_2 by copper/zinc superoxide dismutase (Cu/ZnSOD; *Sod1*) located in the

intermembrane space and cytosol. Mitochondrial H_2O_2 can then diffuse into the nucleus-cytosol. If H_2O_2 encounters a reduced transition metal or is mixed with $O_2 \cdot^-$, the H_2O_2 can be further reduced to hydroxyl radical (OH·), the most potent oxidizing agent of the ROS (**Figure 1**). ROS can damage cellular proteins, lipids, and nucleic acids. Hence, excessive mitochondrial ROS production can exceed the antioxidant defenses of the cell, and the cumulative damage can ultimately destroy the cell by necrosis or apoptosis.

Reactive nitrogen species (RNS) can be generated by mitochondria. At high oxygen tensions, COX (complex IV) uses O_2 as its terminal electron acceptor. However, at low oxygen tension, complex IV can reduce nitrite to nitric oxide (NO). In yeast, the mitochondrially produced NO then induces an alternative nuclear complex IV subunit, COX5b, which enhances complex IV NO production (128, 129). NO is also generated from arginine and O_2 by the three types of nitric oxide synthase (NOS): neuronal, inducible, and endothelial (130–132).

NO is a vasodilator. Hence, NO production by complex IV provides an oxygen-based feedback loop. When oxygen tension is low, complex IV switches to NO synthesis. NO diffuses out of the mitochondrion and cell, causing relaxation of the vascular smooth muscle cells that results in vasodilation (133). This in turn increases tissue oxygenation. However, this system can be compromised by genetic defects that inhibit the ETC and increase ROS production, given that mitochondrial $O_2 \cdot^-$ reacts with NO to generate peroxynitrite, which is inactive as a vasodilator. This may be the reason for the stroke-like episodes associated with the MELAS mtDNA *tRNA^{Leu(UUR)}* nucleotide 3243A>G mutation (34), leading to the proposal that MELAS strokes could be treated with arginine supplementation (134).

Peroxynitrite is a potential reactant that can oxidize reduced groups such as Fe-S centers and sulfhydryls. Peroxynitrite also reacts with tyrosines in proteins to produce nitrotyrosines (nitrosylation), which can affect protein structure and function (135).

The Mitochondrial Permeability Transition Pore

The mitochondria contain a self-destruct system, the mtPTP. The mtPTP is activated when the biochemical health of the mitochondria and cell declines, specifically when mitochondrial energy production declines, ROS generation increases, and excessive Ca^{2+} is released into the cytosol and taken up by the mitochondria. When the mtPTP is activated, it opens a channel in the mitochondrial inner membrane, short-circuits ΔP, and initiates programmed cell death (apoptosis). Activation of the mtPTP can be inhibited by cyclosporin A (1).

The opening of the mtPTP and the collapse of ΔP are associated with the release of cytochrome *c*, procaspase-9, apoptosis-initiating factor, and endonuclease G from the mitochondrial intermembrane space into the cytosol. The former two interact with cytosolic Apaf1 to form the apoptosome, activating the caspase cascade that degrades the cellular proteins. The latter two are transported to the nucleus and degrade the chromatin. Cells with defective mitochondria are thus degraded from the inside out, stopping the release of the mitochondrial bacteria–like antigens into the circulation (1).

Mitochondrial and Cellular Redox Biology

Mitochondrial redox reactions are generally considered in the context of generating ATP by OXPHOS. However, mitochondrial redox chemistry is of profound importance to the redox balance of the entire cell and is central to the signal transduction pathways that regulate nuclear gene expression (11, 136, 137). As a result, the use of therapeutics from the perspective of increasing ATP production by feeding more electrons into the ETC when the ETC is inhibited by a genetic defect simply increases the cellular reduction state and increases ROS production. Similarly, attempting to limit mitochondrial ROS production by providing excessive antioxidant compounds may disrupt ROS signaling pathways essential for cell growth. For these reasons, attempts to treat mitochondrial

NOS: nitric oxide synthase

diseases by metabolic supplementation without consideration of the complex network of cellular redox reactions have generally resulted in either disappointing or counterproductive results. Therefore, for us to address mitochondrial metabolic therapy, it is essential that we first discuss the cellular redox chemistry that would be influenced by the treatments.

Redox regulation of the epigenome. The direct link between the mitochondrial redox state and the regulation of cellular energy metabolism is evident from the biology of the sirtuins. Mammals possess seven sirtuins: Sirt1 and -2 located in the nucleus and cytosol; Sirt3, -4, and -5 in the mitochondrion; and Sirt6 and -7 in the nucleus (121). Sirt1 regulates both mitochondrial biogenesis, through the FOXOs and PGC-1α, and mitophagy, through deacetylation of members of the Agt family of proteins (138). Sirt3 deacetylates various OXPHOS proteins and also modulates the mitochondrial metabolic rate (139).

However, redox signaling's role in regulation of the epigenome is much more pervasive and more ancient than that of the sirtuins. Energy, in the form of reducing equivalents, flows through biological systems from reduced (calorie intake) to oxidized (oxygen). Thus, the use of oxygen as the terminal electron acceptor makes a wide range of redox potentials available to the eukaryotic cell, permitting a broad range of protein modulation through alterations in thiol/disulfide ratios, ROS oxidation, and RNS nitrosylation.

Redox control systems. ROS participate in the activation of a wide spectrum of tyrosine and serine/threonine kinases including Src kinase, protein kinase C (PKC), mitogen-activated protein kinase (MAPK), receptor tyrosine kinases, etc. (123–127). Moreover, ROS also modulate the activity of phosphatases such as protein tyrosine phosphatase 1B, Src homology 2–domain-containing tyrosine phosphatase 2, and phosphatase and tensin homolog, which modulate phosphoprotein levels (140). Mitochondrial and cellular

redox states also determine the redox ratios of the nucleus-cytosolic and mitochondrial NADPH/NADP$^+$, nucleus-cytosolic and mitochondrial NADH/NAD$^+$, thioredoxins 1 and 2 (SH)$_2$/SS [Trx1(SH)$_2$/SS and Trx2(SH)$_2$/SS, where SH stands for thiol and SS for disulfide], gamma-glutamylcysteinylglycine [reduced glutathione/oxidized glutathione (GSH/GSSG)], and cysteine/cystine (CyS/CySS). These eight key redox nodes coordinate the regulation of a broad range of processes. They are also coupled to each other and to a wide range of redox-sensitive proteins.

Within the mitochondrion, the NADPH/NADP$^+$ redox state is coupled to that of Trx2(SH)$_2$/SS and to GSH/GSSG by thioredoxin reductase 2$^{Oxd/Red}$ (TrxR2$^{Oxd/Red}$) and glutathione reductase$^{Oxd/Red}$ (GSHR$^{Ox/Red}$). Similarly, in the cytosol NADPH/NADP$^+$ is coupled to Trx1(SH)$_2$/SS and to GSH/GSSG by TrxR2$^{Oxd/Red}$ and by GSHR$^{Ox/Red}$ (127, 140–143).

The above redox control nodes regulate ROS levels and alter the thiol/disulfide redox status of multiple proteins. Consequently, changes in the redox potential of the control nodes provide a powerful system through which a wide range of signal transduction pathways and transcription factors can be coordinated (127, 140–143).

The importance of these redox control nodes for cell growth, differentiation, and death is clear from the consequences of inactivation of the associated genes in the mouse. Glutathione is synthesized in the cytosol by the condensation of cysteine and L-glutamate to generate gamma-glutamylcysteine, catalyzed by gamma-glutamylcysteine synthetase, the rate-limiting step. Glutathione synthesis is completed by the addition of glycine to the C terminus by glutathione synthetase. Mice that have been mutated to lack the gamma-glutamylcysteine synthetase [also known as glutamylcysteine ligase (GCL)] catalytic subunit (*Gclc*) experience early embryonic lethality. The mutant embryos show increased apoptosis, and the *Gclc* null blastocyst cells cannot grow in a GSH-free medium unless supplemented with N-acetylcysteine

(144, 145). Liver-specific inactivation of mouse *Gclc* results in hepatic steatosis, inflammation, increased lipid peroxidation, and marked changes in mitochondrial morphology and function (146). Mice lacking the GCL modifier subunit (*Gclm*) are viable and fertile, but they have reduced GSH levels, and their *Gclm* null fetal fibroblasts are more sensitive to oxidative stress (147).

The importance of the thioredoxin redox system for cell survival, growth, and differentiation is demonstrated by the genetic inactivation of the mouse Trx1(SH)$_2$/SS, Trx2(SH)$_2$/SS, TrxR1$^{Oxd/Red}$, and TrxR2$^{Oxd/Red}$ genes. Targeted disruption of the Trx1(SH)$_2$/SS gene resulted in early embryonic lethality (148). The absence of mitochondrial Trx2(SH)$_2$/SS in mouse also causes early embryonic lethality associated with massive apoptosis in the embryo and in nonviable embryonic fibroblasts (149).

Targeted depletion of TrxR1$^{Oxd/Red}$ results in early embryonic lethality and nonviable embryonic fibroblasts, although cardiomyocytes are not affected (150). The inactivation of the mitochondrial TrxR2$^{Oxd/Red}$ in mouse also causes early embryonic lethality, with increased liver apoptosis, reduced hematopoietic differentiation, heart defects with abnormal heart mitochondria, and slow-growing embryonic fibroblasts (151).

The critical role of the thiol/disulfide redox control systems stems from the fact that many proteins harbor redox-sensitive cysteines. These thiols can become oxidized to disulfides by reacting with another cysteine thiol within the protein to generate a disulfide bridge, or with a free cysteine to generate protein-CyS-SCy (cysteinylation) or with the thiol in glutathione to generate protein-CyS-SG (glutathionylation). These thiol-disulfide reactions are mediated by the redox status of 2GSH/GSSG, 2CyS/CySS, Trx1(SH)$_2$/SS, and Trx2(SH)$_2$/SS. The redox potential of each of these redox control nodes is a function of the steady-state redox status of the couples and the concentrations of their reactants and products: $E_h = E_o + RT/NF \ln[SS/(SH)^2]$, where E_h and E_o stand for actual and steady-state redox

potential, respectively. Hence, each redox control node can have different redox potentials and act on different proteins to regulate their redox state and thus activity (127, 140–142). Factors that modulate these redox nodes include the availability of reducing equivalents (calories), oxidative stress, and intracellular and extracellular environmental influences (**Figure 2**).

Each cellular compartment maintains discrete redox nodes at the potential necessary to perform that compartment's function. GSH/GSSG redox control nodes are found in the mitochondrion, peroxisome, cytosol, endoplasmic reticulum, lysosome, nucleus, and extracellular space. CyS/CySS nodes are important in the cytosol, lysosome, and extracellular space. Trx2(SH)$_2$/SS control nodes function exclusively in the mitochondrion, whereas Trx1(SH)$_2$/SS control nodes function in the cytosol and the nucleus (**Figure 2**). The overall hierarchy of redox control node potentials from reduced to oxidized is: mitochondria, cytosol, nucleus, endoplasmic reticulum, extracellular space. This hierarchy follows from the flow of reducing equivalents into the mitochondria via the ETC and into the cytosol through glycolysis, the pentose phosphate shunt, and the mitochondria.

Within the mitochondrion, reducing equivalents are maintained on the soluble carriers, NAD$^+$ and NADP$^+$. Hence, the concentration and redox status of these electron carriers set the upper limits of electronegativity for the pathways to which they contribute electrons. The redox status of the mitochondrial redox couples has been estimated to be (*a*) −405 mV for NADPH/NADP$^+$, (*b*) −340 to −360 mV for Trx2(SH)$_2$/SS, (*c*) −300 mV for GSH/GSSG, and (*d*) −250 mV for NADH/NAD$^+$ (140–142, 152, 153). In the cytosol, the potentials of the redox couples have been estimated to be (*a*) −393 mV for NADPH/NADP$^+$, (*b*) −280 mV for Trx1(SH)$_2$/SS, (*c*) from −260 to −170 mV for GSH/GSSG, and (*d*) −160 mV for CyS/CySS. In the nucleus, the redox status of Trx1(SH)$_2$/SS is −300 mV. The redox state of the endoplasmic reticulum is set by
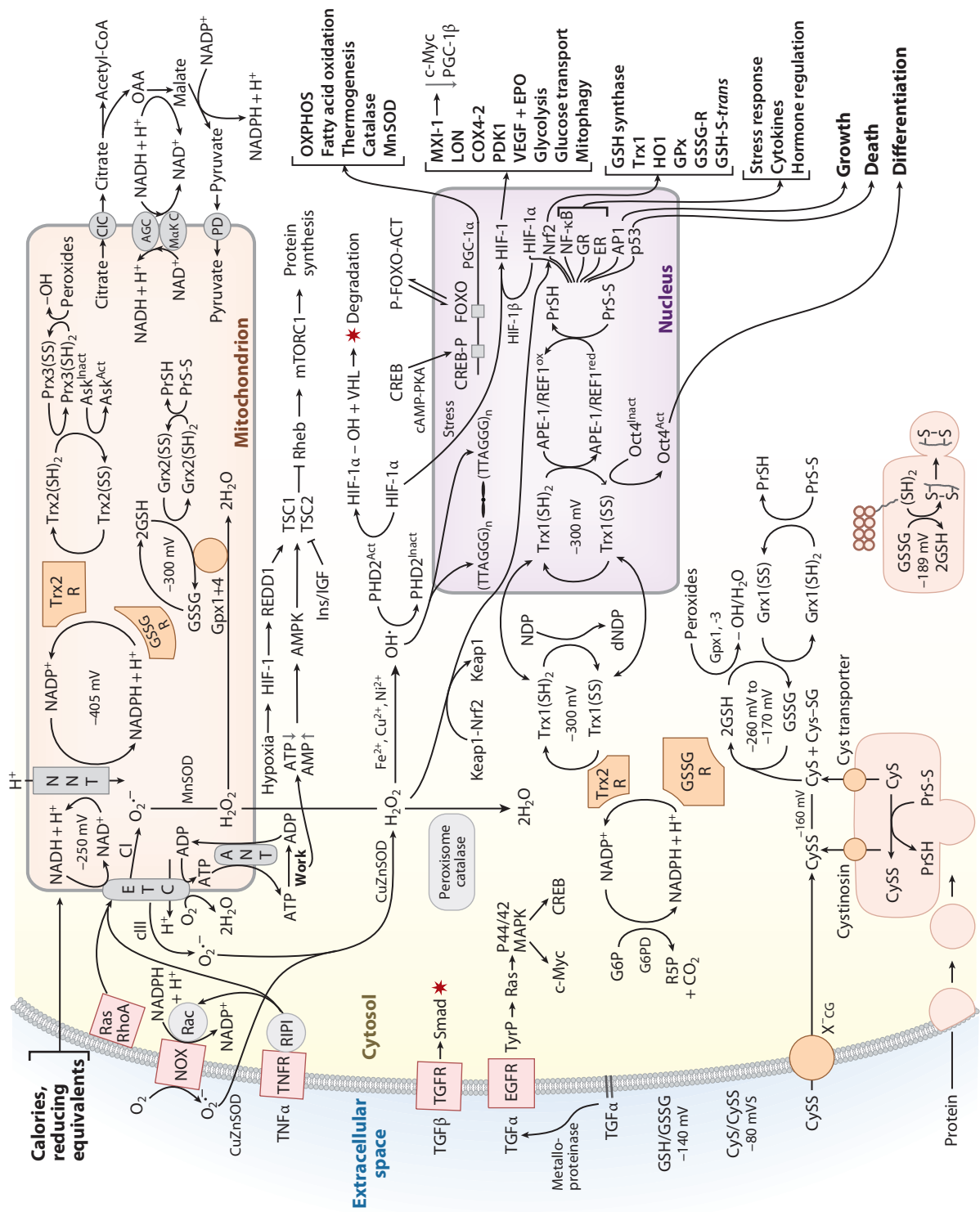
GSH/GSSG at -189 mV. The plasma (extra-cellular space) redox control nodes for healthy individuals have been estimated to be -140 mV for GSH/GSSG and -80 mV for CyS/CySS (**Figure 2**) (140–142, 152, 153).

In the mitochondrion (**Figure 2**), reducing equivalents from dietary calories are converted to NADH and fed into the ETC through complex I. Because the redox potential of NADH/NAD$^+$ within the mitochondrion is -250 mV, most of the electrons from NADH flow down the ETC to O$_2$ at $+1200$ mV to generate H$_2$O. However, approximately 1%–5% of the electrons of the ETC are transferred directly to O$_2$ to generate O$_2\cdot^-$. The chemical affinity of O$_2$ for electrons is unchanged by oxygen tension, and the K_M (Michaelis constant) for O$_2$ by complex IV places a lower limit on the reduction of O$_2$ by complex IV. As a result, as O$_2$ concentration declines, the ETC becomes reduced and mitochondrial ROS production increases.

Mitochondrial H$_2$O$_2$ levels are regulated by the glutathione peroxidases and the peroxiredoxins. These in turn derive their reducing potential from NADPH. Generation of NADPH from NADH requires additional reducing potential. This is provided by the mitochondrial nicotinamide nucleoside transhydrogenase (NNT), which uses the mitochondrial inner-membrane ΔP to provide the necessary energy to transfer the reducing equivalents from NADH to NADP$^+$, increasing the reducing potential from -250 mV (NADH/NAD$^+$) to -405 mV (NADPH/NADP$^+$). NADPH has sufficient reducing potential to convert GSSG to 2GSH via the mitochondrial GSHR$^{Ox/Red}$ and also to


Figure 2

Oxidation-reduction (redox) regulation of the epigenome. The cellular redox system is coordinated by eight redox control nodes: mitochondrial and cytosolic NADPH/NADP$^+$, mitochondrial and nucleus-cytosolic NADH/NAD$^+$, thioredoxins 1 and 2 (SH)$_2$/SS [Trx1(SH)$_2$/SS and Trx2(SH)$_2$/SS, where SH stands for thiol and SS stands for disulfide], reduced glutathione/oxidized glutathione (GSH/GSSG), and cysteine/cystine (CyS/CySS). In the mitochondrion, a small portion of the electron transport chain (ETC) electrons are directly transferred to O$_2$ to generate superoxide anion (O$_2\cdot^-$), which is converted to hydrogen peroxide (H$_2$O$_2$) by manganese superoxide dismutase (MnSOD) in the matrix or by copper/zinc superoxide dismutase (Cu/ZnSOD) in the cytosol. The mitochondrial H$_2$O$_2$ level is regulated by the GSH-driven glutathione peroxidases 1 and 4 (Gpx1 and -4), and by the Trx2(SH)$_2$-driven peroxiredoxins 3 and 5 (Prx3 and -5). Both GSH and Trx2(SH)$_2$ are reduced from NADPH, which is generated from NADH by the mitochondrial nicotinamide nucleoside transhydrogenase (NNT). H$_2$O$_2$ can diffuse into the cytosol-nucleus, where it can be detoxified by peroxisomal catalase. H$_2$O$_2$ can also be converted to hydroxyl radical (OH$\cdot$) when it encounters a reduced transition metal. Reactive oxygen species (ROS), O$_2\cdot^-$, H$_2$O$_2$, and OH$\cdot$ can alter redox potentials of Trx1(SH)$_2$/SS, Trx2(SH)$_2$/SS, GSH/GSSG, and CyS/CySS, which regulate the activities of redox-sensitive proteins containing cysteines. ROS can also react with the guanine-cytosine (GC)-rich chromosomal telomere repeats, causing their premature shortening. On the plasma membrane, NADPH oxidases (NOX) can also generate ROS to produce a bactericidal oxidative burst or to stimulate cell growth or mediate cell death. The cytosolic and nuclear Trx1(SH)$_2$/SS can act through apurinic/apyrimidinic endonuclease/redox factor 1 (APE-1/ Ref1$^{Red/Ox}$) to regulate activator protein 1 (AP1, c-Jun), hypoxia-inducible factor 1 (HIF-1), nuclear factor–E2 related factor 2 (Nrf2), nuclear factor kappa B (NF-κB), p53, glucocorticoid receptor (GR), and estrogen receptor (ER). AP1 regulates DNA replication and cell growth, HIF-1 modulates glycolytic and oxidative energy metabolism, Nrf2 regulates cellular stress response genes, NF-κB regulates cytokines production and the inflammatory response, and p53 modulates cell death. Trx1(SH)$_2$/SS also regulates the activity of the stem cell pluripotency factor, Oct4. GSH/GSSG maintain the redox potential of the endoplasmic reticulum for the proper processing of secreted proteins. CyS/CySS play an important role in the lysosomal hydrolysis of cysteine-rich proteins containing disulfide bridges. Extracellular CyS/CySS and GSH/GSSG can also regulate cell growth and proliferation through epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK). Abbreviations: AMPK, AMP-activated protein kinase; ANT, adenine nucleotide translocator; COX, cytochrome c oxidase; CREB, cAMP response element binding; dNDP, deoxynucleoside diphosphate; EPO, erythropoietin; FOXO, forkhead box, subgroup O; HO, heme oxygenase; IGF, insulin-like growth factor; LON, mitochondrial LON protease; mTORC, mammalian target of rapamycin; NADH, reduced nicotinamide adenine dinucleotide; NDP, nucleoside diphosphate; OAA, oxaloacetic acid; OXPHOS, oxidative phosphorylation; PDK, pyruvate dehydrogenase kinase; PGC-1β, peroxisome proliferator–activated receptor gamma coactivator 1 beta; PHD, prolylhydroxylase domain protein; PKA, protein kinase A; PrSS, protein disulphide; PrSH, protein thiol; TGF, transforming growth factor; TGFR, TGF receptor; TNF, tumor necrosis factor; TNFR, TNF receptor; TSC, tuberous sclerosis protein; VEGF, vascular endothelial growth factor; VHL, von Hippel–Lindau tumor suppressor.

convert Trx2(SS) to Trx2(SH)$_2$ via the mitochondrial TrxR2$^{Red/Ox}$ (**Figure 2**).

The concentration of peroxide within the mitochondrial matrix is regulated by the GSH-driven glutathione peroxidases (Gpxs), specifically Gpx1 and -4, and by the Trx2(SH)$_2$/SS-driven peroxiredoxins (Prxs) Prx3 and -5. The mitochondrial Gpx system reduces H$_2$O$_2$

to 2H$_2$O by a $2e^-$ transfer from 2GSH to produce GSSG. Because glutathione cannot be synthesized by the mitochondrion, it must be imported from the cytosol by the mitochondrial inner-membrane dicarboxylate and 2-oxoglutarate transporters (127, 141). Trx2(SH)$_2$/SS, acting through mitochondrial Prx3, can reduce a variety of mitochondrial
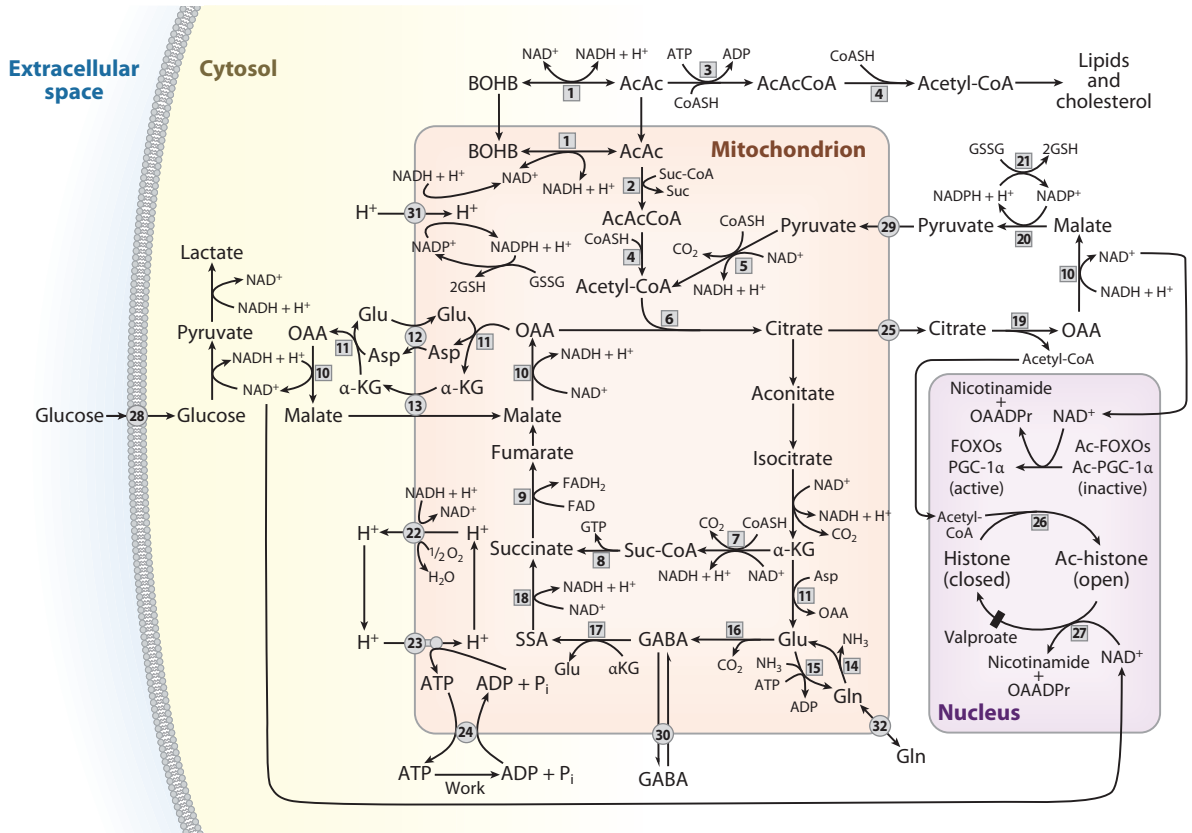
1 β-hydroxybutyrate dehydrogenase	14 Phosphate-dependent glutaminase	26 HAT
2 Succinyl-CoA:3-ketoacid transferase	15 Glutamine synthetase	27 HDAC, sirtuin
3 AcAcCoA synthetase	16 Glutamate decarboxylase	28 Glucose transporter (GLUT)
4 AcAcCoA thiolase	17 GABA transaminase	29 Monocarboxylate transporter
5 Pyruvate dehydrogenase	18 Succinic semialdehyde dehydrogenase	30 GABA transporter (not characterized)
6 Citrate synthetase	19 ATP-citrate lyase	31 NNT
7 α-KG dehydrogenase	20 Malic enzyme	32 Glutamine transporter (hypothetical)
8 Succinyl-CoA synthetase	21 Glutathione reductase	
9 Succinate dehydrogenase	22 ETC	
10 Malate dehydrogenase	23 H$^+$-translocating ATP synthase	
11 Aspartate aminotransferase	24 Adenine nucleotide translocator	□ Enzyme
12 Aspartate/glutamate carrier	25 Citrate carrier	○ Membrane transporter
13 Malate/α-KG carrier		
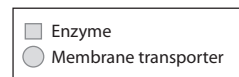
peroxides (H_2O_2, alkyl hydroperoxide, and peroxynitrite) (**Figure 2**). Inactivation of *Prx3* in the mouse results in animals that are grossly normal but whose macrophages show significantly higher ROS production. However, upon lipopolysaccharide (LPS) exposure, the mutant mice develop severe injury to lung DNA and proteins (154). Thus, mitochondrial H_2O_2 production is directly regulated by the redox potential of the NADPH/NADP$^+$ couple, which in turn is maintained by the flow of reducing equivalents through the mitochondrial ETC and the ΔP. The Trx2(SH)$_2$/SS redox state can also stabilize the mtPTP (140, 155). Finally, the mitochondrial GSH/GSSG redox control node acts through glutaredoxin 2 (Grx2) to regulate the function of a variety of proteins through their thiols (156).

Mitochondrial NADH/NAD$^+$ redox levels are coupled to the cytosol through the mitochondrial inner-membrane NADH/NAD$^+$ shuttle systems. The malate-oxaloacetate shuttle involves the aspartate-glutamate carrier and the malate-alpha-ketoglutarate carrier linked to cytosolic and mitochondrial malate dehydrogenase and aspartate aminotransferase (**Figures 2** and **3**). Although the redox statuses of the mitochondria and cytosol are coupled, the redox potential of the two NADH/NAD$^+$ systems can differ due to differences in concentrations of NAD$^+$ in the two compartments and differences in the activities of the carriers.

The nucleus and cytosol also rely on the NADPH/NADP$^+$ redox couple, with its redox potential of -393 mV, to reduce Trx1(SH)$_2$/SS. However, the cytosolic NADPH/NADP$^+$ redox potential is physically separated from the mitochondrial NADPH/NADP$^+$ redox couple. Hence, the cytosolic NADPH must be generated within the cytosol. One of the processes by which mitochondrial redox potential can be transferred to the cytosol is via the export of mitochondrial citrate. Within the mitochondrion, acetyl-CoA is condensed with oxaloacetate by citrate synthase to generate citrate. The citrate can then be exported to the cytosol by the citrate carrier. In the cytosol, citrate can be cleaved by ATP-citrate lyase to generate

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Figure 3

Metabolic, redox, and epigenomic influences of the ketogenic diet. Calories can enter the cell as glucose or as ketones [beta-hydroxybutyrate (BOHB) and acetoacetate (AcAc)]. Glucose is processed through the cytosolic glycolytic pathway to generate cytosolic NADH (reduced nicotinamide adenine nucleotide) and pyruvate. Pyruvate can enter the mitochondrion and be processed into acetyl-CoA, CO_2, and mitochondrial NADH. Ketones are directly metabolized by the mitochondrion to generate acetyl-CoA and mitochondrial NADH, leaving cytosolic NAD$^+$ oxidized. Ketone body–derived acetyl-CoA is condensed with oxaloacetic acid (OAA) to generate citrate. In inhibitory neurons, citrate is converted to alpha-ketoglutarate (α-KG), then glutamate (Glu), and then gamma-aminobutyric acid (GABA). Increased GABA inhibits neuronal excitation, suppressing seizures. Increased acetyl-CoA also drives the tricarboxylic acid (TCA) cycle to generate mitochondrial NADH, which is oxidized by the electron transport chain (ETC) to increase ΔP and drive ATP synthesis. The elevated ATP drives the neuron plasma membrane K$^+$ and Ca^{2+} pumps, thereby stabilizing the membrane potential, decreasing depolarization, and inhibiting seizures. The increased ΔP also drives the mitochondrial inner-membrane nicotinamide nucleotide transhydrogenase (NNT) to convert NADH to NADPH. The elevated NADPH drives the reduction of mitochondrial oxidized glutathione (GSSG) to reduced glutathione (GSH), protecting the mitochondria against oxidative stress. Mitochondrial citrate can be exported to the cytosol, where it is cleaved by ATP-citrate lyase to generate cytosolic acetyl-CoA and OAA. The OAA is reduced by malic dehydrogenase to malate, and the malate is oxidized to pyruvate, generating NADPH. The cytosolic NADPH can then reduce cytosolic GSSG to GSH, protecting the cytosol against oxidative stress. Cytosolic acetyl-CoA can drive histone acetylation by histone acetylases (HATs), opening chromatin and activating transcription of energetic genes. Histone deacetylases (HDACs) can reverse this acetylation, inhibiting transcription of energetic genes. HDAC inhibitors such as valproate keep the chromatin open and energy production high. However, valproate treatment of patients with severe energy deficits may drive energy production beyond the capacity of the impaired bioenergetic system, resulting in failure and pathology (306). Oxidation of ketones by the mitochondria leaves cytosolic NAD$^+$ oxidized. This activates Sirt1 to deacetylate the forkhead box, subgroup O (FOXO) and peroxisome proliferator–activated receptor gamma coactivator 1 alpha (PGC-1α) transcription factors, resulting in the induction of mitochondrial biogenesis, oxidative phosphorylation (OXPHOS) and ATP production. Abbreviations: AcAcCoA, acetoacetyl-CoA; Suc-CoA, succinyl-CoA; CoASH, coenzyme A; Asp, aspartate; Gln, glutamine; SSA, succinic semialdehyde; Ac-histone, acetylated histone; FAD, flavin adenine dinucleotide; OAADPr, O-acetyl-ADP-ribose.

acetyl-CoA and oxaloacetate. The oxaloacetate can then be reduced to malate by malate dehydrogenase and NADH. Malate is then converted to pyruvate by malic enzyme, reducing $NADP^+$ to NADPH in the process. The pyruvate can either return to the mitochondrion to generate acetyl-CoA or be reduced to lactate in the cytosol by lactate dehydrogenase via NADH (**Figures 2** and **3**). The generation of NADPH via malic enzyme was recently found to be central to cancer cell metabolism, mediated by the c-myc induction of the glutaminolysis pathway (157–159). Therefore, mitochondrial redox reactions play an important role in maintaining the cytosolic NADPH/$NADP^+$ redox ratio.

Cytosolic NADPH can also be generated by the pentose shunt. Glucose is phosphorylated to glucose-6-phosphate by ATP and hexokinase. Glucose-6-phosphate dehydrogenase then converts glucose-6-phosphate + H_2O + $2NADP^+$ to ribose-5-phosphate + $2NADPH + CO_2 + 2H^+$.

In the nucleus and cytosol, reducing equivalents flow from NADPH/$NADP^+$ through $GSHR^{Ox/Red}$ and $TrxR1^{Red/Ox}$ to regulate the cytosolic GSH/GSSG and $Trx1(SH)_2$/SS ratios. The GSH/GSSG redox potential regulates (*a*) Gpx1 and Gpx3 to control peroxides and (*b*) Grx1 to regulate the redox state of proteins (**Figure 2**). Grx1-deficient mice are prone to myocardial injury as a result of ischemic reperfusion insult and to hyperoxia-induced lung injury, and *Grx1* null myocyte-enhancing factors are sensitive to paraquat but are more resistant to tumor necrosis factor alpha (TNFα) and actinomycin D–induced apoptosis (160).

The $Trx1(SH)_2$/SS redox potential acts through Prx1 and -2 to reduce peroxides (**Figure 2**). *Prx1* mutant mice have shortened life spans due to hemolytic anemia, cancer, and reduced natural killer cell number and function. Also *Prx1* null myocyte-enhancing factors proliferate slowly and are more sensitive to oxidative stress (161). Mice lacking *Prx2* generate markedly increased levels of ROS and are more susceptible to paraquat-induced oxidative stress. They develop splenomegaly

and hemolytic anemia, their macrophages are more susceptible to oxidative stress, and their hearts are sensitive to ischemia-reperfusion injury (162–164).

$Trx1(SH)_2$/SS redox potential also regulates apoptosis signal–regulating kinase (ASK1). ASK1 is a MAPK that activates the c-Jun N-terminal kinase (JNK) and p38 MAPK pathways and is required for TNFα-induced apoptosis. Under reducing conditions, ASK1 is complexed with $Trx(SH)_2$. However, under oxidizing conditions presumably generated by ROS and GSSG, $Trx(SH)_2$ is converted to the oxidized form [$Trx1(SS)$], and ASK1 is released. The resulting activated ASK1 then initiates the stress-response system and apoptosis (165, 166). Because TNFα preferentially oxidizes the mitochondrial $Trx2(SH)_2$/(SS), rather than the nucleus-cytosol $Trx1(SH)_2$/(SS) (167), it is possible that ASK1 interacts with both $Trx2(SH)_2$ and $Trx1(SH)_2$ (140, 141).

The $Trx1(SH)_2$/SS redox potential also acts through the bifunctional protein apurinic/apyrimidinic endonuclease/redox factor 1 (APE-1/ $Ref1^{Red/Ox}$). $Ref1^{Red/Ox}$, in turn, regulates activator protein 1 (AP1, c-Jun), nuclear factor–E2 related factor 2 (Nrf2), nuclear factor kappa B (NF-κB), p53, glucocorticoid receptor, estrogen receptor, and hypoxia-inducible factor 1 alpha (HIF-1α) by maintaining conserved cysteine residues in the reduced form required for DNA binding. AP1 is also activated by oxidant induction of JNK, which phosphorylates critical domains in the c-Jun transactivation domain. Activation of the AP1 system directly regulates an array of genes involved in cell growth and development (**Figure 2**). Nrf2, which regulates stress-response genes, is maintained in an inactive state in the cytosol in a complex with the Kelch-like ECH-associated protein 1 (Keap 1). Oxidation of a key cysteine in Keap 1 releases Nrf2 to migrate to the nucleus, where it binds to its antioxidant-response elements. This process induces a wide range of antioxidant and stress-response genes, including many of those for glutathione biology (**Figure 2**). NF-κB is also activated by ROS. Physiological stimuli

such as TNFα binding to its receptor increase ROS production, which activates NF-κB. NF-κB then enters the nucleus to induce cytokine and inflammatory gene expression (**Figure 2**) (127, 140, 141, 152, 168). The capacity of the stem cell transcription factor Oct-4 to bind to DNA is also regulated by oxidation and is associated with the binding of Trx to cysteines in the POU domain of the protein. Overexpression of Trx(SH)$_2$/SS increases the transcriptional activity of Oct-4, whereas expression of an inactive Trx(SH)$_2$/SS inhibits its transcriptional activity (169).

Studies of the Trx1(SH)$_2$/SS, GSH/GSSG, and CyS/CySS redox control nodes of the cytosol have revealed that the GSH/GSSG redox state can vary depending on the cell status and environmental conditions. The human colon carcinoma cell line, HT29, actively proliferates when the cellular GSH/GSSG redox ratio is −260 mV, but when induced to differentiate through use of butyrate or isothiocyanate, the redox state shifts to −200 mV (170). Exposure of HT29 cells to an extracellular CyS/CySS ratio of −75 mV activates NF-κB and induces mitochondrial apoptosis (142). Exposure of murine lung fibroblasts to a CyS/CySS redox potential of −46 mV stimulates their proliferation and expression of fibronectin, NF-κB, and transforming growth factor beta 1 (TGFβ1) (171). Different cell types can respond to the same redox signal differently, presumably due to differences in the signal transduction pathways. Thus, exposing cells to external redox signals can cause the cells to grow, differentiate, or die by changing their internal cellular redox status.

Trx1(SH)$_2$/(SS) is also an important redox control node for the nucleus. However, its redox potential in the nucleus is less variable than that of cytosolic Trx1(SH)$_2$/(SS), remaining close to −300 mV (**Figure 2**) (141, 142). The redox potential of the endoplasmic reticulum is maintained by GSH/GSSG and is relatively oxidized. This is important in the processing of secreted proteins (**Figure 2**) (141). The redox status of the peroxisome has not been

determined. However, the peroxisome encompasses a variety of active redox reactions and antioxidant enzymes such as catalase (141).

In the lysosome, the potential of the CyS/CySS redox control node is thought to play an important role in the lysosomal hydrolysis of cysteine-rich proteins containing disulfide bridges (152). The leftover cystine can be transported out of the lysosome by cystinosin (*CTNS*), a proton-cystine symporter. Cysteine may be transported out of the lysosome by the e and f lysosomal transport systems and can be actively taken up by the lysosome via the cysteine transporter (172). In the lysosome, cysteine may act as a reductant to counteract the increased oxidation state of accumulated cystine (**Figure 2**). Cystine can also be taken up by the cell through the plasma membrane transport system CG (X$^-_{GC}$) carrier, and in the cytosol it is reduced to cysteine by GSH. In cystinosis patients who lack *CTNS* (173), cystine accumulates in the lysosomes and ultimately precipitates as crystals. The exceedingly high concentration of cystine in the cystinosis lysosomes may result from a combination of cystine generated from protein degradation and from the import of cytosolic cysteine, which balances the lysosomal redox state. The resulting depletion of the cytosolic cysteine could inhibit cytosolic glutathione synthesis and deplete cellular GSH, thus increasing cellular oxidative stress, mitochondrial dysfunction (174–178), and apoptosis (167, 179–181), all of which are seen in cystinosis cells and patients. Inactivation of *Ctns* in mice also results in lysosomal development of cystine crystals, as well as other aspects of the cystinosis phenotype (182). Cystinosis in human and mouse can be treated by cysteamine. Cysteamine is imported into the lysosome, where it generates a cysteine-cysteamine mixed disulfide; the mixed disulfide is then exported from the lysosome by the lysine transporter (183).

The redox status of the extracellular space (plasma) is determined by the GSH/GSSG and CyS/CySS redox control nodes. In normal individuals, the redox potential of plasma GSH/GSSG is approximately −140 mV, and

that for CyS/CySS is approximately -80 mV (**Figure 2**) (184).

Nox reactive oxygen species. Because mitochondrially generated H_2O_2 was probably one of the first molecules used for signal transduction between the protomitochondrion and the proto-nucleus-cytosol, it would have been employed for regulating development in multicellular organisms. Hence, ROS signaling would have become important for cellular growth and differentiation in tissues and organs, even in contexts wherein the mitochondrial redox state could not change. Consequently, an alternative method for developing $O_2 \cdot^-$, the Nox and Doux system, evolved. The Nox proteins may have evolved early in multicellular organisms, as they have been found in chordates, plants, fungi, and myxomycetes (185).

The human genome contains seven members of the Nox family: Nox1–5 and Doux1–2. The Nox enzymes use NADPH and H^+ to reduce O_2 to $O_2 \cdot^-$. The $O_2 \cdot^-$ is then converted to H_2O_2 by Cu/ZnSOD. Because the Nox/Doux enzymes are transplasma or vacuolar membrane proteins, the Cu/ZnSOD required to convert Nox $O_2 \cdot^-$ to H_2O is probably the extracellular SOD isoform (*Sod3*). The Douxs differ from the Noxs in having an additional peroxidase-like ectodomain.

The best-characterized Nox is Nox2 (also known as gp91^{phox}), the enzyme responsible for the bactericidal oxidative burst generated within phagocytic vesicles of macrophages. The phagocyte NADPH oxidase is a multi-subunit enzyme encompassing gp91^{phox}. The membrane-bound Nox2 polypeptide encompasses two hemes, a flavin adenine dinucleotide, and an NADPH-binding site. Nox2 is tightly associated with a second membrane subunit, p22^{phox}, and together these two contain all of the catalytic sites. Activation of Nox2 also requires four soluble cytosolic polypeptides: p40^{phox}, p47^{phox}, p67^{phox}, and the small GTPase Rac. Rac is complexed with the cytosolic RhoGDI. Once activated, Rac is released, and the four cytosolic subunits bind to gp91^{phox} + p22^{phox}. NADPH + H^+ are then oxidized on the cy-

tosolic side of the membrane; the electrons are transferred through the membrane and used to reduce O_2 to $O_2 \cdot^-$ on the outside of the membrane (**Figure 2**) (185).

Whereas Nox2 is important in innate immunity, the other Noxs are found in different tissues and have different functions. Nox1 is found in lung, vascular endothelium, intestinal epithelium, stomach, colon, uterus, pancreas, and prostate. It is induced in a number of tumors and can stimulate cellular replication through ROS production (186). Nox3 is important in inner-ear octoconia formation, Nox4 is important in kidney, and Nox5 is expressed in T- and B-lymphocytes (185).

Rac, the regulator of Nox1, is a member of the Ras family of small GTPases, which regulate ROS production in response to various stimuli. Evidence suggests that Ras-driven ROS production occurs through both Nox1 activation and mitochondrial ROS production. The Ras GTPases can activate PI3K, which generates lipid products to activate GTPase-exchange factors. These factors in turn activate Rac, which stimulates Nox1 to generate ROS (187). Rac can also stimulate NOS, and NO can react with cysteines to regulate protein function through S-nitrosylation (188).

Rac has also been shown to connect ROS production with cytoskeletal remodeling in cultured podocytes. Angiotensin II activation of its type 1 receptor activates Rac, which stimulates Nox1 to generate ROS. The increased ROS stimulates the G-coupled receptor kinase 2, which then phosphorylates the ezrin/radixin/moesin proteins. This process promotes cortical F-actin remodeling, thus tying ROS directly to cytoskeletal organization. TNFα-induced ezrin/radixin/moesin protein phosphorylation in endothelial cells has also been associated with cytoskeletal changes (189). Thus, ROS is directly linked to the regulation of the cytoskeleton.

TNF interaction with tumor necrosis factor receptor 1 (TNFR1) has been found to activate the receptor-interacting protein kinase (RIP1). RIP1 is known to activate NF-κB and MAPK, but it also stimulates the formation

of a complex including Nox1, tumor necrosis factor receptor–associated death domain protein, Nox organizing protein 1, and Rac, resulting in ROS production and necrosis. RIP1 has also been reported to move to the mitochondrion and reduce the interaction between ANT and cyclophilin D (190, 191). The Src tyrosine kinase has also been shown to increase cellular ROS production by phosphorylating the RAC GTPase-exchange factor Vav2. Phosphorylated Vav2 converts the inactive Rac-GDP to the active Rac-GTP, which in turn activates Nox1 to generate ROS (192).

The chronic production of ROS by the Noxs can result in the accumulation of cellular oxidative damage. Hence, ROS from both the Nox/Doux proteins and the mitochondria may contribute to age-related diseases (193). However, mitochondrial $O_2 \cdot^-$ generation must be much more important in cell toxicity than the Noxs, given that inactivation of the *Sod2* gene is perinatal lethal (194, 195) and that inactivation of *Sod1* (196) or *Sod3* is fully compatible with life (197).

The importance of the Nox1 activation and ROS production in the action of such diverse signaling systems as Src and Ras induction of growth and TNF induction of cell death demonstrates the importance of ROS as a fundamental signal transduction system. Therefore, the Nox system confirms the importance of the mitochondrion and associated redox systems in regulation of the eukaryotic epigenome.

Reactive oxygen species, hypoxia, and stress. The regulation of cellular ROS production levels by the mitochondria and the NADPH oxidases can have a range of effects, depending on the level of ROS production and the regulatory proteins expressed in the cells involved (122–127, 140). One of the key regulatory circuits responding to changes in oxygen tension is the HIF-1 system. HIF-1 is composed of two subunits, HIF-1α and HIF-1β. Both are synthesized constitutively, but the turnover of HIF-1α is regulated by O_2 concentration. At high O_2 levels, prolines 402 and/or 562 are hydroxylated by prolylhydroxylase

domain protein 2 (PHD2). Hydroxylation promotes the binding of the von Hippel–Lindau protein, leading to ubiquitination and degradation of HIF-1α. Hence, under normoxic conditions, HIF-1 is inactivated. Under hypoxic conditions, by contrast, O_2, which is a substrate for PHD2, becomes limiting. Moreover, the reduced O_2 stimulates complex III of the ETC to produce ROS, which reacts with the Fe(II) in the PHD2 catalytic center, thereby causing its inactivation. This reaction stops the hydroxylation of HIF-1α by PHD2, blocks von Hippel–Lindau protein binding, and stabilizes HIF-1α and thus HIF-1 (**Figure 2**).

Stabilized HIF-1 binds to the core DNA target (5′-RCGTG-3′) in a large number of genes and activates the genes permitting adaptation to low oxygen tension. Selected genes will be induced, such as genes that enhance glycolysis, including glucose transporters and glycolytic genes such as lactate dehydrogenase (*LDH*); genes to increase vascularization, including erythropoietin (*Epo*) and vascular endothelial growth factor (*VEGF*); and a variety of others (**Figure 2**) (198–200). Induction of HIF-1 also induces PDH kinase 1 (PDK1), which phosphorylates the E1α subunit of PDH, resulting in PDH inactivation. Inactivation of PDH blocks the metabolism of pyruvate to acetyl-CoA, thus inhibiting the flow of reducing equivalents through the TCA cycle to NADH and the mitochondrial ETC, the resulting reduced electron density of the ETC reduces mitochondrial ROS production, and the cytosolic pyruvate and NADH are converted to lactate by LDH (**Figure 3**) (199, 201–203). Activated HIF-1 also induces synthesis of the complex IV COX4-2 subunit as well as the mitochondrial LON protease. The LON protease in turn degrades the normoxic subunit, COX4-1. As a result, the kinetics of complex IV is altered to sustain ATP production at low oxygen tension (200, 204). HIF-1 also inhibits c-myc transcription through induction of MXI-1 and inactivates the c-myc protein. Because c-myc drives the expression of PGC-1β, which induces mitochondrial biogenesis in a variety of tissues (205), inhibition of c-myc transcription

and inactivation of c-myc protein suppress mitochondrial biogenesis. Lastly, HIF-1 activation induces the expression of mitochondrial autophagy protein, BNIP3. BNIP3 antagonizes the binding of Beclin-1 to Bcl2 or Bclx, thus releasing Beclin-1 to initiate mitochondrial autophagy (mitophagy). This release leads to a rapid reduction in mitochondrial number and function (206). Thus, hypoxic activation of HIF-1, mediated by mitochondrial ROS production, reduces mitochondrial function in four ways: COX4 subunit switching, PDH inactivation by phosphorylation, inhibition of mitochondrial biogenesis by suppression of c-myc, and increase of mitochondrial degradation by mitophagy (207).

In addition to O_2 tension, other environmental factors can alter the redox status of the mitochondrial and nucleus-cytosol control nodes. Certain heavy metals selectively alter the redox status of Trx1(SH)$_2$/SS and Trx2(SH)$_2$/SS. Exposure of HeLa cells to Cu, Fe, and Ni results in significant oxidation of the GSH/GSSG redox node, with relatively little effect on Trx1(SH)$_2$/SS and Trx2(SH)$_2$/SS redox states. By contrast, HeLa exposure to As and Hg has little effect on the GSH/GSSG redox state and partially increases the oxidation state of the cytosolic Trx1(SH)$_2$/SS (20 to 40 mV), but it markedly increases the oxidation state of the mitochondrial Trx2(SH)$_2$/SS (>60 mV). Cu, Fe, and Ni contribute to the generation of OH· through Fenton-type reactions, whereas As and Hg disrupt mitochondrial redox homeostasis (208).

Bacterial LPS (*Escherichia coli* O111:B6) injected intraperitoneally into mice is cleared by the liver, which responds with the synthesis and release of inflammatory cytokines such as TNFα and interleukin-1β. These cytokines act on the lung, resulting in acute injury. Within 2 h, plasma CyS levels decline and CySS levels increase, resulting in overall oxidation of the CyS/CySS couple by 10 mV. During the same period, the lung fluid CyS/CySS couple is oxidized by 50 mV. Because cysteine is more prone to H_2O_2 oxidation than glutathione, the changes in plasma CyS/CySS redox

state may reflect acute increases in ROS production (209). The relationship between LPS, TNFα, and oxidative stress is demonstrated in human macrophages derived from the THP-1 leukemia cell line. LPS binds to the Toll-like receptors, activating their tyrosine kinase activity, which in turn activates NADPH oxidase to produce ROS. The ROS then activates the ROS-sensitive PKC, PI3K, MAPK, and tyrosine kinases, leading to induction of HIF-1α transcription. The increased ROS also activate mammalian target of rapamycin and S6 kinase to increase HIF-1α protein synthesis. Finally, the increased HIF-1 induces transcription of inflammation and wound-healing genes. The fact that activation of Nox increases extracellular $O_2\cdot^-$ production could partially explain the rapid oxidation of the plasma CyS/GySS pool (210). Thus, exposure to LPS results in rapid oxidation of both cytoplasmic and plasma redox pools.

Reactive oxygen species, telomeres, and replicative senescence. The importance of redox status for mammalian cell growth and differentiation has been repeatedly demonstrated by the effects of growing cells in different oxygen concentrations. Traditionally, mammalian cells have been cultured in air containing 21% O_2. However, arterial blood has an oxygen concentration of approximately 12%, tissue oxygen levels are approximately 3%, and the mean oxygen level in the brain is approximately 1.5% (211). Growth of mammalian cells in ambient O_2 has been shown to inhibit the differentiation of stem cells (212–216). For example, the differentiation of cultured mouse myogenic C2C12 cells into myotubes at 20% O_2 is enhanced by addition of the ROS trapping agent phenyl-N-*tert*-butylnitrone and inhibited by addition of H_2O_2. Skeletal muscle myoblasts isolated from *Gpx1*$^{-/-}$ mice also show impaired myotube formation (215).

An important DNA target of ROS damage is the chromosomal telomere. Telomeres cap the ends of chromosomes and consist of thousands of copies of the TTAGGG repeat. Telomeres can be eroded during cell replication because

unidirectional DNA replication necessitates the use of Okazaki fragments to initiate lagging strand replication. Consequently, lagging strand replication is always tens of nucleotides short of the chromosomal end. Telomere erosion is countered by telomerase, which uses an RNA template to extend the telomere repeats, thus compensating for the loss of repeats of the lagging stand. Telomerase consists of the RNA template and the reverse transcriptase apoprotein (TERT). Expression of the *TERC* gene for the RNA template is constitutive, but expression of the *TERT* gene is regulated, as the template is high in immortal germ line cells, pluripotent stem cells, and cancer cells but low or absent on terminally differentiated cells. *TERT* and *TERC* mutations are associated with dyskeratosis congenital and pulmonary fibrosis (217).

The ends of chromosomes are capped by a telomere loop (t-loop) and a six-protein complex, shelterin. The shelterin complex blocks the activation of the DNA-repair protein ATM, which is activated by double-strand breaks, and/or ATR, which is activated by single-strand breaks, that would be initiated by free telomere ends. Activated ATM or ATR phosphorylates Chk1 and Chk2, which enforce G1-S and G2-M arrest. They also activate p53 to induce p21, inhibit Cdk, and block the cell cycle (218). When sufficient telomere repeats are lost, the t-loop does not form. This activates the DNA-damage system, thereby activating p53 and causing cell-cycle arrest and cell death by apoptosis (219, 220).

Although telomerase is essential for maintaining telomeres during normal replication, telomeres are sufficiently long that many rounds of DNA replication would be required for incomplete lagging strand replication to erode the telomeres sufficiently to destabilize the t-loop and cause cell arrest and death. Yet telomere erosion is relatively rapid in telomerase-deficient cells such as human diploid fibroblasts. Moreover, if end replication were the primary reason for telomere erosion, then postmitotic cell telomeres would be completely stable. This discrepancy was resolved upon the discovery that the rate of telomere decay correlated with the oxygen tension at which cells were grown (221). It is now clear that telomere decay is primarily due to mitochondrially generated ROS, which cause oxidative damage in the telomere repeats (222–229).

The direct connection between telomere stability and mitochondrial ROS production was revealed following the discovery that, when ROS production increases in cells expressing telomerase, TERT is induced to translocate out of the nucleus and into the mitochondrion (230). Apparently, the increased ROS activates a Src family tyrosine kinase, located in the nuclear periphery, that phosphorylates tyrosine 707 of TERT. Tyrosine 707 signals, through a Ran GTPase–dependent process, for TERT to be exported out through the nuclear pores (231, 232). Once the TERT protein is in the cytosol, an N-terminal mitochondrial targeting peptide directs it into the mitochondrion (230). Within the mitochondrion, TERT reduces mitochondrial ROS production, protects mtDNA from damage, and regulates apoptosis in a Bcl-2-dependent fashion. Although mitochondrial TERT protects the mitochondrion and mtDNA, it does not protect the telomeres. Hence, oxidative damage to the telomeres results in rapid loss of blocks of telomere repeats (232–234). Therefore, the replicative life span of human diploid fibroblasts, the Hayflick limit (219, 220), is presumably due to mitochondrial ROS production and not the end-replication problem.

CyS/CySS and GSH/GSSG in health. Extracellular redox status, mediated by exogenous CyS/CySS and GSH/GSSG ratios, can modulate critical cysteines in extracellular signal transduction molecules (143). These extracellular signals can then modify intracellular redox control nodes to regulate cellular growth, differentiation, and death (127, 140–143).

Growth of human colon cancer Caco-2 cells increases twofold as the CyS/CsSS redox potential is reduced from 0 mV to −150 mV. This growth occurs because exposure of Caco-2 cells to the CyS/CySS

−150 mV reducing environment activates extracellular metalloproteinases that contain redox-sensitive cysteines. The activated metalloproteinases cleave and release cell surface–tethered TGFα. This binds to and activates the epidermal growth factor receptor (EGFR) tyrosine kinase. Activation of the EGFR results in an 80% increase in phosphorylation of EGFR and an increase in phosphorylation of p44/42 MAPK, which activates c-myc and CREB, promoting cell growth and proliferation (**Figure 2**) (235, 236).

Conversely, human plasma GSH/GSSG and CyS/CySS ratios provide a window into an individual's cellular health. In normal individuals, the plasma redox status for both CyS/CySS and GSH/GSSG declines with age. The CyS/CySS redox ratio declines continuously at approximately 0.2 mV per year, starting at −80 mV at age 18 and declining through age 93. GSH/GSSG redox status remains stable at approximately −140 mV until age 45, after which it declines at roughly 0.7 mV per year. Thus, the range of CyS/CySS and GSH/GSSG redox potentials for healthy individuals is −80 to −62 mV and −140 to −119 mV, respectively. By contrast, individuals with a variety of nutritional deficiencies or degenerative conditions have much more oxidized CyS/CySS and GSH/GSSG redox potentials on the order of −20 mV and −80 mV, respectively. This has been observed in individuals who are depleted of antioxidants or dietary cysteine, who abuse alcohol or smoke, or who have type 2 diabetes or myocardial perfusion defects—all of which impinge on mitochondrial function (142).

MITOCHONDRIAL METABOLIC THERAPEUTIC APPROACHES

Realization of the complex interactions between (*a*) mitochondrial redox biology and energetics and (*b*) cellular signaling and nuclear gene expression provides some appreciation for why treatment of patients with single mitochondrial metabolites or cofactors has yielded inconsistent results.

Metabolic Therapeutics

Current pharmacological or metabolic therapeutics have focused on increasing mitochondrial ATP production, reducing the mitochondrial ROS produced, modifying Ca^{2+} uptake and toxicity, stabilizing the mtPTP, and inducing mitochondrial biogenesis (**Table 2**). Ironically, one of the oldest therapeutic approaches—fasting and the ketogenic diet—remains the most promising treatment for mitochondrial defects. Hence, understanding the manifold potential actions of fasting and the ketogenic diet may point the way toward more comprehensive mitochondrial therapies.

Neutraceuticals to enhance mitochondrial function. A variety of metabolic strategies to increase mitochondrial energy production have been attempted. However, to be effective, these therapies must be tailored to the patient's specific mitochondrial defect. In contrast, treatments of patients with so-called mitochondrial cocktails have met with mixed results.

Coenzyme Q₁₀ and analogs. CoQ is the most commonly recommended metabolic therapy for patients with putative mitochondrial disease (**Table 2**). Its advantages are that it is generally nontoxic and that it may both increase electron entry into the mitochondrial ETC and function as an antioxidant. Numerous open-label clinical trials have been performed with CoQ with varying success.

In instances where the patient has a primary defect in CoQ synthesis, CoQ supplementation can largely alleviate symptoms. However, primary CoQ deficiency resulting from defects in one of the several steps involved in the CoQ biosynthesis is rare (237). Primary CoQ deficiency syndromes are also heterogeneous, presenting with encephalomyopathy, Leigh syndrome, severe multisystem infantile disease, cerebellar ataxia, or pure myopathies (238). Fortunately, as soon as an accurate diagnosis has been made, the patient is very likely to benefit from CoQ supplementation.

Table 2 Metabolic and pharmacological agents used to treat mitochondrial disease[a]

Agent	Indication	Mechanism	Patient dose (per day)	Reference(s)
Cofactors that increase the production of ATP				
CoQ	Mitochondrial disorders	Transfers electrons from complex I and II to complex III	Variable, 5 mg kg^{-1}	307, 308
Idebenone	Mitochondrial disorders	Transfers electrons from complex I and II to complex III	Variable; child, 5 mg kg^{-1} day^{-1}; adult, 90 mg day^{-1} (up to 225 mg day^{-1})	237, 309
Succinate	Complex I deficiency	Bypasses complex I	6 g day^{-1}	244
Dimethylglycine	Mitochondrial disorders	Transfers electrons to CoQ	5 mg kg^{-1} if >33kg, 50 mg kg^{-1} if <33 kg	310
Other cofactors and vitamin or metabolic supplements				
Thiamine (vitamin B1)	PDH deficiency	Cofactor of PDH	Variable: child, 5 mg day^{-1}; adult, 90 mg day^{-1} (up to 225 mg day^{-1})	249, 250
Riboflavin (vitamin B2)	Complex I and II deficiency	Increases complex I and II activities	100 mg day^{-1}	289
Nicotinamide (vitamin B3)	MELAS	Increases nicotinamide adenine dinucleotide	50 mg kg^{-1} day^{-1}	311
Dichloroacetate	Severe lactic acidosis	Reduces lactic acidosis, stimulates PDH	50 mg kg^{-1}	120, 256, 312, 313
Creatine monohydrate	Mitochondrial myopathy	Increases muscle phosphocreatine	10–20 g day^{-1}	314, 315
Carnitine	Carnitine deficiency	Increases carnitine level	3 g day^{-1}	258
L-arginine	MELAS	Increases nitric oxide production	0.15–0.3 g kg^{-1} day^{-1}	258
Reactive oxygen species scavengers and mitochondrial antioxidants				
CoQ/idebenone	Mitochondrial disorders	Act as antioxidant	Variable; child, 5 mg kg^{-1} day^{-1}; adult, 90 mg day^{-1} (up to 225 mg/day)	237, 307–309
Vitamin E	Mitochondrial disorders	Antioxidant	50 μg day^{-1}	–
Vitamin C (ascorbate)/ vitamin K3 (menadione)	Mitochondrial disorders	Antioxidants, bypass complex III	4 g day^{-1}/40 mg day^{-1}	245–247
Lipoic acid	Pyruvate dehydrogenase deficiency	Acts as antioxidant, stimulates PDH	600 mg day^{-1}	261
Triacetyluridine	Mitochondrial disorders	De novo pyrimidine biosynthesis	Animal models	255
Modulation of the mtPTP				
Cyclosporin A	–	Inhibits the mtPTP	In vitro studies	255
Nortriptyline	–	Inhibits the mtPTP	Animal models	269
Regulation of mitochondrial biogenesis				
Sirtuin analogs	Mitochondrial disorders	Increase mitochondrial biogenesis	Animal models	273
Bezafibrate	Mitochondrial disorders	Increases mitochondrial biogenesis	In vitro studies, animal models	276

(*Continued*)

Table 2 (*Continued*)

Agent	Indication	Mechanism	Patient dose (per day)	Reference(s)
Exercise therapy and diet				
High-fat diet	Mitochondrial disorders	Increases electron transfer	50%–60% calories	289
Ketonic diet	Intractable seizures, mitochondrial disorders	Bypasses glycolytic pathway	4:1 lipid:nonlipid ratio	100, 290
Endurance exercise	Mitochondrial myopathy	Aerobic training induces OXPHOS	–	280, 281
Resistance exercise	Mitochondrial myopathy	Activation and proliferation of satellite cells in muscle, shifting of heteroplasmy	–	282, 283

[a]Abbreviations: CoQ, coenzyme CoQ_{10}; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes; mtPTP, mitochondrial permeability transition pore; OXPHOS, oxidative phosphorylation; PDH, pyruvate dehydrogenase.

CoQ and idebenone, a short-chain quinone that readily crosses the blood-brain barrier, have also been used to treat a wide spectrum of other mitochondrial diseases, but with varying degrees of success (239, 240). High doses have been used to treat Alzheimer disease and Friedreich's ataxia (238). Friedreich's ataxia is an autosomal recessive disease that manifests with hereditary ataxia in association with diabetes mellitus and hypertrophic cardiomyopathy. Idebenone has been reported to counteract the cardiomyopathy of Friedreich's ataxia patients (241, 242), and high doses of this quinone have also been reported to afford some neurological protection (243). However, Friedreich's ataxia patients need to be treated as early as possible to slow down the disease process before definitive mitochondrial damage occurs.

Succinate plus CoQ have been used to treat a CPEO patient with respiratory failure that was predominantly due to a complex I defect. The logic of this therapy was that reducing equivalents could be introduced into the ETC via succinate through complex II, thus bypassing the complex I block. The CoQ would then enhance the transfer of the electrons from complex II to complex III (244).

A similar bypass strategy has been reported for the treatment of a complex III defect in a patient harboring a cytochrome *b* nonsense mutation. This patient was treated with ascorbate (vitamin C) plus menadione; the logic was that the menadione would transfer reducing equivalents directly to complex IV (245, 246). This treatment significantly improved the patient's muscle strength and mitochondrial energy production, as assessed by ^{31}P magnetic resonance spectroscopy (247).

Other cofactor and vitamin supplements. Various other combinations of vitamins and metabolic supplements have also been used to treat mitochondrial disease patients; see **Table 2** (240, 248). Thiamine has been used with success in children with PDH deficiency due to alterations within the thiamine binding site of the X-linked PDH-E1α subunit gene (249). Also, an adult patient with peripheral neuropathy and optic neuropathy associated with a PDH deficiency showed improvement in neurological symptoms and visual acuity with 1 g day^{-1} of thiamine (250). A thiamine deficiency can cause severe neurological degenerative disease and thus is a useful model of neurodegenerative disease (251).

Primary carnitine deficiency due to defects in the carnitine transporter, OCTN2, can present with hypoketotic hypoglycemia and hepatic encephalopathy or with skeletal and cardiac myopathy. This disease is effectively

treated with free carnitine (252). Carnitine levels are also frequently decreased in patients with other mitochondrial disorders, due to the excretion of unoxidized acylcarnitines. These patients may also benefit from oral L-carnitine supplementation at 3 g day^{-1} of free carnitine (253).

Uridine. OXPHOS is necessary for de novo pyrimidine biosynthesis because dihydroorotate CoQ oxidoreductase (dihydroorate dehydrogenase) uses the ETC as an electron sink via CoQ. Therefore, patients with mitochondrial defects can become pyrimidine deficient and may benefit from exogenous uridine (254). Evidence that this approach may be clinically beneficial comes from rodents poisoned with MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) that were partially protected by treatment with triacetyluridine, the bioavailable form of uridine. MPTP is a dominanergic neuron-specific complex I inhibitor that kills cells in the basal ganglia, resulting in movement disorders in humans and rodents (255).

Dichloroacetate. Dichloroacetate has been proposed as a treatment for mitochondrial diseases because it inhibits PDK. PDK inactivates PDH through phosphorylation of the E1α subunit. The resulting activation of PDH should drive pyruvate into the mitochondrion, increasing mitochondrial acetyl-CoA and NADH levels and potentially increasing energy production. However, when patients who have severe neonatal lactic acidosis due to PDH deficiency or who harbor the 3243 A>G mutation were treated with dichloroacetate, only limited success in ameliorating clinical symptoms was observed (120, 256, 257). Moreover, a high incidence of drug-induced peripheral neuropathy was reported in a group of 3243A>G patients treated with dichloroacetate (120).

L-arginine. L-arginine has been proposed as a treatment for MELAS 3243A>G syndrome patients during the acute stroke phase. The logic of this approach is that the stroke-like episodes of MELAS syndrome are the result of mitochondrial production of $O_2 \cdot^-$, which destroys NO in vascular endothelial and smooth muscle cells. Because NO is a vasodilator and the vasoconstrictor is a peptide, loss of NO results in chronic vasoconstriction and transient ischemia. As the substrate of NOS, L-arginine should stimulate NOS to generate more NO and alleviate the vasoconstriction. L-arginine is administrated either orally or by intravenous injections between crises at 0.5–0.3 g kg^{-1} day^{-1}. Although preliminary reports indicate suppression of the stroke-like episodes with L-arginine therapy (258), these claims need to be validated by a standardized clinical trial.

Reactive oxygen species scavengers and mitochondrial antioxidants. Mitochondrial oxidative damage could be ameliorated by natural (lipoic acid, vitamins C and E, CoQ, etc.) or experimental synthetic (MnTDEIP, EUK134, etc.) antioxidants. These compounds either provide an electron sink (vitamins C and E and CoQ) or catalytically degrade the ROS (MnTDEIP, EUK134, etc.).

It is generally believed that CoQ has significant antioxidant properties (239). Although vitamins C and E should have a similar capacity, they have given mixed results in both individual patients and broad-based epidemiological studies (240). These discrepancies may relate to the action of the various compounds on the different cellular compartments (**Figure 2**) (136). To enhance efficacy, these compounds have been covalently linked to the lipophilic triphenylphosphonium cation. The positive charge of this cation is electrostatically attached to the negatively charged mitochondrial matrix, directing the antioxidant to this compartment. This approach has proven promising in model-system studies (259, 260).

Lipoic acid, a coenzyme of the PDH complex, is also an antioxidant. A lipoic acid dose of 600 mg day^{-1} has been shown to decrease several markers of oxidative stress in plasma, including low-density lipoprotein oxidation and urinary isoprostanes in healthy subjects (261).

Metabolic antioxidants have the advantage that they are relatively nontoxic, perhaps because the cell brings them back into redox homeostasis with the rest of the cellular compartments, thus minimizing perturbation of existing cellular signaling networks (**Figure 2**). However, they have the disadvantage of being only transiently effective at compensating for the severe mitochondrial ROS production that results from mitochondrial OXPHOS defects.

The efficacy of antioxidant therapy can be greatly enhanced by introducing catalytic antioxidants into the mitochondrial matrix. This has been demonstrated by creating a transgenic mouse in which the normally peroxisomal catalase was redirected into the mitochondrial matrix by removal of the C-terminal peroxisomal targeting sequence and addition of an N-terminal mitochondrial targeting sequence. The resulting transgenic mice have an increased life span and reduced mtDNA somatic mutation rate, and they are also more resistant to oxidative challenge and to cardiomyopathy (262).

This benefit has been observed in mouse model systems of mitochondrial disease. These mice were treated with catalytic chemical antioxidants, which remove ROS without themselves being destroyed. One class of these compounds is MnSOD and catalase mimetics. Intraperitoneal injection of MnTBAP [Mn(III)tetrakis (4-benzoic acid) porphyrin chloride] into mice deficient in the MnSOD rescued the mice from their lethal neonatal cardiomyopathy. Also, the MnTBAP partially rescued the mouse tissues from the superoxide destruction of the Fe-S center–containing enzymes, including mitochondrial aconitase and complexes I, II, and III. However, MnTBAP does not readily cross the blood-brain barrier, and the MnTBAP-treated animals developed movement disorders in association with a spongiform encephalopathy (263). Treatment of the MnSOD-deficient animals with the salen Mn complexes EUK-8, -134, and -189, which do cross the blood-brain barrier, protected these animals from both the cardiomyopathy and the spongiform encephalomyopathy (264). Although treatment of cells and animals with

mitochondrially targeted antioxidants may appear to be a promising approach for treatment of a wide variety of clinical problems associated with excessive mitochondrial ROS production, the complete elimination of mitochondrial ROS production could disrupt critical cellular redox signal transduction systems and thus be deleterious (**Figure 2**). Because mitochondrial ROS is an important mitogen, highly effective catalytic antioxidants may inhibit embryonic development and thus act as a teratogen or inhibit adult stem cell replication, leading to long-term toxicity. Hence, more information about the role of mitochondrial ROS and redox in the cellular signal transduction pathways will be required before optimal therapeutic doses of antioxidant can be selected.

Modulation of the mitochondrial permeability transition pore. Although the accumulation of mtDNA damage that leads to the erosion of mitochondrial energy production has been proposed to be the pathophysiological basis of mitochondrial disease and aging, it is the loss of cells due to necrosis and apoptosis that leads to organ failure, clinical symptoms, and ultimately death. This cell death is precipitated by the activation of the mtPTP and initiation of apoptosis (1). Therefore, blocking of the activation of the mtPTP should prolong the life of the patient's cells, slow the rate of onset, and diminish the severity of the symptoms (265).

It has long been known that activation of the mtPTP can be inhibited by cyclosporin A. However, cyclosporin A is also an immunosuppressant, so it is not a feasible drug for long-term treatment of mitochondrial disease patients. Analogs of cyclosporin A, now in development, inhibit the mtPTP but lack its immunosuppressive activity. These analogs show great promise for treating mitochondrial patients (265). A particularly exciting demonstration of the potential power of these mtPTP inhibitors is in the treatment of Bethlem myopathy and Ullrich congenital muscular dystrophy. Mutations in the collagen VI gene result in a progressive myopathy. Surprisingly, the clinical features of these

diseases can be markedly reduced simply by treatment with mtPTP inhibitors (266, 267), which not only demonstrates the value of modulating the mtPTP in therapeutics but also indicates that mitochondrial dysfunction and the activation of the mtPTP are relevant to a much wider range of clinical problems than simple mitochondrial disorders.

Cyclosporin A exposure and Bcl-2 overexpression have also been shown to stabilize the mtPTP in cybrid cell lines carrying LHON mtDNA mutations that cause complex I defects (268). Nortriptyline, a tricyclic antidepressant, was found to be protective in a mouse model of amyotrophic lateral sclerosis. It was shown to inhibit the activation of the mtPTP and thus to delay the onset of symptoms and increase the life span of the mice (269). Therefore, modulation of the mtPTP may have broad implications for treating mitochondrial disease.

Modulating Ca^{2+} toxicity. Increases in cytosolic Ca^{2+} are buffered by the mitochondrial uptake of the Ca^{2+} at the expense of the mitochondrial inner-membrane ΔP. Mitochondrial Ca^{2+}, in turn, is a major regulator of TCA enzyme activity and ATP production (270). Mitochondrial defects can reduce the capacity of the mitochondria to take up Ca^{2+}, and the reduced mitochondrial ATP can diminish the activity of ion transporters, including Ca^{2+} pumps. Decreased ΔP and increased cytosolic Ca^{2+} could then increase the probability of mtPTP activation and apoptosis. Therefore, perturbation of the mitochondrial regulation of Ca^{2+} could also contribute to the pathophysiology of mitochondrial disease.

Transmitochondrial cybrid cell lines harboring the pathogenic mtDNA MERRF $tRNA^{lys}$ 8344 A>G mutation, but not the mtDNA NARP 8993A>G mutation, show aberrations in cellular Ca^{2+} signaling (271). Hence, loss of Ca^{2+} homeostasis should be addressed in certain mitochondrial disorders.

Regulation of mitochondrial biogenesis. Caloric restriction has been shown to increase mitochondrial biogenesis and longevity in a variety of animal models; it acts in part through the sirtuins, such as Sirt1 (272, 273). Therefore, mitochondrial energy output may be enhanced in patients through activation of Sirt1 by use of resveratrol or its analogs (SRT1720, SRT2183, etc.). The success of this approach has already been reported for several age-related diseases, including type 2 diabetes (274).

Resveratrol, a Sirt1 activator extracted from grape skins, induces mitochondrial biogenesis and aerobic capacity in mice through deacetylation and activation of PGC-1α. These processes protect the mice against diet-induced obesity and insulin resistance (273). Moreover, upregulation of PGC-1 coactivators stimulates mitochondrial OXPHOS in cells harboring mtDNA mutations and thus may provide therapeutic benefits for mtDNA disorders (275).

Overexpression of PGC-1α has also been found to delay the onset of symptoms and increase the life expectancy in a mouse model of muscle COX deficiency, generated by the muscle-specific inactivation of *COX10.* Bezafibrate, a known activator of the PPAR/ PGC-1α pathway, had a similar effect (276). Bezafibrate has also been shown to increase several mitochondrial functions, including enzyme activities and protein levels in mitochondrial defective cell lines (277, 278). Therefore, modulation of the PPAR/PGC-1α pathway and administration of bezafibrate represent promising new therapeutic strategies for the treatment of patients with mitochondrial disorders.

Modulation of mitochondrial turnover. Manipulation of mitochondrial autophagy (mitophagy) may also be an important therapeutic approach. Mitophagy removes damaged or mutated mitochondria (279). It has been hypothesized that the accumulation of somatic mtDNA mutations observed during the aging process may be due to a decline of the mitophagy process. However, excessive mitophagy could deplete mitochondrial levels and thus be deleterious. Approaches that may permit beneficial modulation of autophagy include

treatment with PI3K antagonists and agonists (279) or treatment with Sirt1 agonists (138).

Exercise therapy and diet. Mitochondrial diseases are generally associated with exercise intolerance, and exercise training is beneficial to the treatment of mitochondrial diseases. Muscle deconditioning in response to inactivity and unloading is common in patients with mitochondrial disease. Promotion of aerobic training with moderate exercise can attenuate or avoid this deconditioning and thus be beneficial to patients (280).

Exercise-based treatment strategies can be subdivided into two categories: endurance exercise and resistance exercise. Endurance training is known to induce OXPHOS and antioxidant capacity and is expected to modulate exercise intolerance in patients. The effects of aerobic training in a group of 20 patients carrying well-characterized mtDNA molecular defects were investigated over a 12-week period, and the approach had beneficial effects (281). In another study (280) of eight patients with single large-scale mtDNA deletions, the subjects showed significant improvement after 14 weeks of aerobic training. Oxygen utilization and skeletal muscle oxygen extraction, peak capacity to do work, and submaximal exercise tolerance were all increased. However, detraining resulted in a loss of these physiological benefits, whereas another round of 14 weeks of training for a subset of patients maintained those individuals' beneficial adaptations (280). Both studies showed that aerobic training is well tolerated and safe for patients, but the investigators observed no change in mtDNA copy number.

Resistance exercise activates the proliferation of satellite cells in skeletal muscle as a consequence of muscle fiber injury. The level of mutant mtDNA in satellite cells is generally lower than in mature muscle fibers. Hence, fusion of satellite cells shifts the heteroplasmic level toward the wild-type mtDNAs, leading to improvement in patient OXPHOS capacity.

In a case study of a patient harboring a tRNA mutation, significant changes in mutant load

and muscle morphology were induced by either eccentric or concentric resistance exercise training (282). In another recent study, a group of eight patients carrying large-scale mtDNA deletions underwent resistance exercise testing during 12 weeks; the patients showed significant therapeutic benefits and a reduced proportion of mutant mtDNAs (283).

As an alternative, although riskier, approach to exercise, skeletal muscle may be partially damaged by bupivacaine injection. This damage could induce satellite cells with reduced mtDNA mutant loads to fuse with the muscle fibers and reduce heteroplasmy (284).

Ketogenic diet. Although each of these approaches has been reported to be of some benefit for mitochondrial disease patients, several of the affected pathways appear to be activated by the ketogenic diet. Therefore, in acquiring an understanding of the mechanisms of action of the ketogenic diet, we could obtain insights into how to design a more systematic approach to treating bioenergetic disease (**Figure 3**).

Since the 1920s, fasting and the ketogenic diet have been known to be one of the most effective approaches for treatment of intractable seizures in children (285). Ketone bodies (beta-hydroxybutyrate and acetoacetate) are generated by the liver during fasting by beta oxidation of fatty acids. Generally, the brain uses glucose as its main energy source, with a ratio of glucose to ketones of approximately 97:3. However, during short-term fasting this ratio can shift to ~70:30, and during starvation it can shift to ~30:70 (286). Caloric restriction has also been shown to prolong life span in several organisms in association with reduced oxidative stress and increased antioxidant enzymes (272). This is the result of shifting metabolism from a more glucose-based glycolytic metabolism to a more fatty acid oxidation–based mitochondrial metabolism (1, 11).

In addition to preventing seizures in children, ketone treatment in adults has been found to (*a*) prevent symptoms of experimental hypoglycemia, including anxiety, fainting,

hunger, irritability, and tremor, and (*b*) inhibit astrocytoma tumor growth and vascularization (286). Beta-hydroxybutyrate also protects cultured mesencephalic neurons from MPP$^+$ (1-methyl-4-phenylpyridinium) toxicity and hippocampal neurons from amyloid beta 42 toxicity (287).

Generally, the ketogenic diet is composed of >90% fat by weight; is low in carbohydrates but adequate in protein, vitamins, and minerals; and is typically calorie restricted by 10%–25% (288). The brains of rats that were fasting or on a ketogenic diet were found to have a seven- to eightfold increase in the uptake of ketones (286). A high-fat diet enhanced with vitamins and CoQ is well tolerated. It has been reported to both provide short-term benefits (289) and improve the outcomes of mitochondrial disorders and intractable epilepsy patients (290).

Although the efficacy of the ketogenic diet is well established for treating childhood seizures that are not responsive to antiepileptic drugs, the mechanism by which ketones ameliorate seizure activity is still actively debated. Current observations may be summarized and understood by a review of the metabolic pathways surrounding beta-hydroxybutyrate and acetoacetate metabolism that are centered largely within the mitochondrion (**Figure 3**). Beta-hydroxybutyrate and acetoacetate are taken up from the blood and are interconverted through beta-hydroxybutyrate dehydrogenase. Within the mitochondrion, beta-hydroxybutyrate and acetoacetate are converted to acetyl-CoA, which condenses with oxaloacetate to generate citrate; the excess of acetyl-CoA depletes oxaloacetate. The citrate is then converted to alpha-ketoglutarate by the TCA cycle, and alpha-ketoglutarate is converted to glutamate by transfer of an amino group from aspartate, which regenerates oxaloacetate. Because oxaloacetate is depleted, glutamate synthesis is favored. The brain glutamate can then be decarboxylated to generate gamma-aminobutyric acid (GABA), an inhibitory neurotransmitter that can suppress neuronal hyperactivity generated by excitatory neurons. The relevance of this mechanism for ketonic diet action has been demonstrated by extensive metabolic studies (291–298) and by the action of the antiepileptic drug vigabatrin, which inhibits the GABA-degrading enzyme GABA transaminase (294).

Increased production of GABA depletes the excitatory neurotransmitter, glutamate, whose release is reduced in cultured glutamatergic neurons exposed to beta-hydroxybutyrate relative to glucose (299). Moreover, in a pentylenetetrazole-induced rat seizure system, a 24-h low-glucose and low-calorie exposure was sufficient to reduce seizure activity. This reduction was associated with increased brain alanine generation and its export across the blood-brain barrier. Because alanine is generated from glucose-derived pyruvate by the transamination from glutamate, the export of brain alanine converts the excitatory neurotransmitter glutamate to alpha-ketoglutarate, thereby reducing neuronal excitation (**Figure 3**) (300).

In addition to enhancing GABA production, the increased acetyl-CoA generated from beta-hydroxybutyrate and acetoacetate also drives the TCA cycle (**Figure 3**). This increases mitochondrial NADH plus H$^+$ and FADH$_2$ production, fueling the ETC, increasing ΔP, and stimulating mitochondrial ATP synthesis by the H$^+$-translocating ATP synthase. The increased ATP is then exported to the cytosol to drive the neuronal Na$^+$ and Ca$^+$ pumps, thus raising the neuronal plasma membrane resting potential and inhibiting the synchronous neuronal discharge characteristic of epilepsy (285). Because beta-hydroxybutyrate is more energy rich than pyruvate (the former has a 31% high caloric density per C$_2$ unit), it produces more ATP (285). Moreover, in the heart, ketone metabolism increases the acetyl-CoA content 9- to 18-fold and citrate levels two- to fivefold; decreases succinate, oxaloacetate, and aspartate two- to threefold; increases the reduction status of NAD$^+$ together with the oxidation status of CoQ; and increases heart hydraulic efficiency by 25% relative to pyruvate (285, 301). The ketogenic diet also

increases hippocampal mitochondrial density and ATP levels in a *Aldh5a1*$^{-/-}$ mouse seizure model, in association with reduced seizures (302). In the hippocampus, the ketogenic diet also results in the coordinated induction of energy metabolism gene transcripts, inducing 33 of the 34 energy metabolism genes (including 21 genes involved in OXPHOS) and 39 of the 42 genes encoding mitochondrial proteins. A ketogenic diet also results in a 46% increase in the mitochondrial density in the hippocampus of animals and increases the phosphocreatine-to-creatine ratio (288).

Ketones have also been reported to reduce cellular and mitochondrial oxidative stress, to increase antioxidant scavenging molecules, and to stabilize the mtPTP. Studies on cultured rat neonatal cortical neurons revealed that beta-hydroxybutyrate and acetoacetate prevented neuronal cell death induced by glutamate. Ketones also significantly reduced mitochondrial production of ROS and associated cytotoxic effects in association with increased ETC oxidation of NADH (303). Rats maintained on a ketogenic diet were found to have a twofold increase in hippocampal mitochondrial GSH levels, which increased the GSH/GSSG redox potential values from -230 mV for control animals to -247 mV for ketogenic diet–fed animals. This finding correlated with a 1.3-fold increase in glutamate cysteine ligase, a 1.9-fold increase in its catalytic subunit, and a 1.6-fold increase in its modulatory subunit. Similarly, the mitochondria-specific coenzyme A/coenzyme A glutathione disulfide redox ratio was significantly increased in the hippocampus. Mitochondria purified from the hippocampus of ketogenic diet–fed rats also showed a reduction in H_2O_2 production and were resistant to the H_2O_2-induced mtDNA damage (304).

Rat neocortical brain slices and neurons exposed to H_2O_2 showed protection against electrophysiological changes, loss of cell viability, and quenching of DCF (2′,7′-dichlorofluorescin) fluorescence when incubated with beta-hydroxybutyrate and acetoacetate, which were also protective against diamide thiol oxidation–induced electrophysiological changes and neuronal cell death. Ketones also directly increased the Ca^{2+} threshold of the mtPTP. The protective effects of ketones on electrophysiological effects were mimicked by the mtPTP inhibitors, cyclosporin A acting on cyclophilin D, and bongkrekic acid acting on ANT (305). Therefore, ketone bodies reduce mitochondrial ROS production, increase antioxidant defenses, and stabilize the mtPTP.

The reduction in mitochondrial ROS production and the stabilization of the mtPTP could also arise from increased mitochondrial OXPHOS. The resulting increased ΔP would also stabilize the mtPTP. The increased GSH/GSSG ratio could be the result of increased mitochondrial NADPH levels generated by the mitochondrial NNT, which in turn is driven by the increased mitochondrial ΔP. Increased mitochondrial NADPH levels would increase the mitochondrial GSH/GSSG ratio. Similarly, the antioxidant defenses in the cytosol could be enhanced by the export of the excess mitochondrial citrate into the cytosol, where it can be cleaved by ATP-citrate lyase into oxaloacetate and acetyl-CoA. The oxaloacetate could then be reduced by malate dehydrogenase, and the malate could be oxidized to pyruvate by malic enzyme, thereby reducing $NADP^+$ to NADPH. The increased cytosolic NADPH could then reduce the cytosolic GSSG to GSH (**Figure 3**).

The induction of mitochondrial bioenergetic genes could be explained by the increased generation of cytosolic acetyl-CoA produced from the mitochondrial citrate cleaved by ATP-citrate lyase. Elevated acetyl-CoA could then drive nuclear histone acetyltransferases to acetylate histones. This process would open the chromatin and increase the expression of the nDNA-encoded mitochondrial genes (**Figure 1**) (121). This speculation is supported by the observation that the antiepileptic drug valproic acid is a histone deacetylase inhibitor. Valproate treatment would increase histone acetylation and perhaps increase mitochondrial gene transcription (**Figure 3**).

Furthermore, during the glycolytic conversion of glucose to pyruvate, cytosolic NAD^+ is reduced to NADH. The high-cytosolic acetyl-CoA generated from the pyruvate via mitochondrial citrate would drive not only histone acetylation but also FOXO and PGC-1α acetylation, inactivating these proteins. Because the Sirt1 deacetylase cannot use NADH as a substrate, the acetylated FOXOs and PGC-1α would remain inactive, thus downregulating mitochondrial biogenesis and OXPHOS and shifting metabolism toward glycolysis. By contrast, oxidation of fatty acids or ketones occurs entirely within the mitochondrion. The mitochondrial acetyl-CoA would still be exported to the cytosol via citrate to acetylate histones and stimulate transcription. However, only the mitochondrial NAD^+ would be actively reduced to NADH, leaving the cytosolic NAD^+ more oxidized. The high-cytosolic NAD^+ levels would then stimulate the Sirt1 deacetylation of the FOXOs and PGC-1α, thus causing the induction of mitochondrial biogenesis and shifting metabolism toward OXPHOS and away from glycolysis (**Figure 3**). These speculations suggest that the ketogenic diet may act at multiple levels: It may decrease excitatory neuronal activity, increase the expression of bioenergetic genes, increase mitochondrial biogenesis and oxidative energy production, and increase mitochondrial NADPH production, thus decreasing mitochondrial oxidative stress.

CONCLUSION

Although it is becoming increasingly clear that mitochondrial dysfunction is a common factor in a wide range of complex diseases, effective therapeutic interventions are still not readily available. New approaches for genetic therapies for nDNA-encoded mitochondrial diseases as well as for mtDNA diseases are beginning to offer alternatives for individuals suffering from these devastating disorders. However, these approaches are not as likely to relieve the devastating symptoms suffered by individuals with bioenergetic diseases. Current metabolic interventions have been consistently disappointing, but new insights into the integrated energetic network of the cell are providing fresh insight into the mode of action of the older therapeutic approaches, such as fasting and the ketogenic diet, and suggesting more integrated approaches for restoring cellular energetics to homeostasis. Therefore, there is hope that more sophisticated and potentially more efficacious therapeutic approaches will evolve from our current knowledge.

SUMMARY POINTS

1. Mitochondrial disorders are a heterogeneous group of multisystem diseases. They can result from mutations in hundreds of genes distributed across all of the chromosomes as well as the mtDNA.

2. mtDNA genetics follows the specific rules of maternal inheritance, heteroplasmy, mitotic and meiotic segregation, and threshold effect.

3. Mitochondrial dysfunction is a common factor in a wide spectrum of metabolic and generative diseases, cancers, and aging.

4. There are two main approaches to mitochondrial disease therapy: genetic and metabolic pharmacological.

5. Progress is being made in developing genetic therapies for mitochondrial disease, although efforts to develop effective metabolic and pharmacological therapies have been surprisingly disappointing. A primary reason for difficulties in developing metabolic and pharmacological therapies is our lack of understanding of the complex bioenergetic and redox networks of the mitochondrion and the cell.

6. Consideration must be given to how the genetic defect and the therapeutic approach perturb the cellular bioenergetic and redox network and how these networks respond to the perturbation. Hence, extensive research on cellular and mitochondrial bioenergetics, redox systems, and mitochondrial-nuclear interactions should be performed.

7. As metabolic and pharmacological therapies are developed for mitochondrial dysfunction, randomized clinical trials of homogeneous patient cohorts must be conducted to ensure that the treatments are not harmful and to affirm their therapeutic benefit.

8. The development of effective metabolic and pharmacological treatments for defined mitochondrial diseases holds promise for future therapies that could ameliorate the symptoms of a broad range of common metabolic and degenerative disorders, cancers, and even aging.

DISCLOSURE STATEMENT

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