Satisfying the Immense Energy Demands of the Body, and the Effects of Mutation within the Mitochondrial ATP6 Gene

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Summary

The cells constituting the human body require immense amounts of energy to power them. Occasionally, defects occur within the powerhouse of the cell, the mitochondria, that lead to severe and often fatal untreatable diseases such as Leigh Syndrome (LS) and Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP). Both LS and NARP result from the T8993G or T8993C mutations within the ATP6 gene in the mitochondrial genome. Because mitochondrial genes are maternally inherited and the cellular effects of mutation are dosage dependent, LS and NARP syndromes have mutant thresholds of 90% to 95% and 70% to 90% respectively. Research within the last fifteen years has linked the mutation to decreased ATP synthase stability, assembly, catalysis, and oxidative stress. This review will focus on ATP6 and the T8993G mutation.

Introduction

The human body and the individual cells that compose it have an immense energy quota to satisfy. Over time, mammalian cells evolved an energy efficient oxidative-phosphoralation strategy for obtaining the ubiquitous energy currency ATP that produces much more energy per molecule of glucose than simple glycolysis. Without ATP, cellular function would cease to exist in our cells. The powerhouse of the eukaryotic cell is the mitochondria, which is a dual-membrane organelle thought to have once been a bacterium before becoming part of the eukaryotic cell through endosymbiosis. The proper functioning of this organelle is critical to the viability of the cell, and if it is defective an array of diseases can manifest.

Two rare but prominent diseases associated with mitochondrial dysfunction are Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP) and Leigh Syndrome. NARP is characterized by the above features as well as developmental impairments, dementia, and sensory deficits (Schon et al., 2001). These patients typically live into their thirties and forties (Manfredi et al., 2002). Leigh Syndrome (LS) is a fatal encephalopathy consisting of neuronal atrophy localized within the brainstem and basal ganglia (Ciafaloni et al., 1993). Individuals with LS die during infancy and exhibit motor and developmental impairments, respiratory deficits, and other features associated with the location of atrophy (Ciafaloni et al., 1993). Neither disease is treatable. In common between these diseases is their underlying cause; a defective ATP6 gene within the mitochondrial genome. This is an essential gene that will kill any organism/model if knocked-out or under-expressed.

It is apparent that the inability to satisfy the body's energy requirements is detrimental, but in LS and NARP syndromes cellular atrophy is specific rather than global. The predominant hypothesis within the field for this specificity has to do with the varying energetic demands of different tissues. The nervous system and the brain, in particular, are by far the greatest consumers of energy within the body, and for good reason. Neurons are constantly active and involved in everything from cognition, sensation, muscle regulation, respiration, etc. Thus, if mutant mitochondria are unable to meet the energy requirements of neurons but could still provide enough for other cell types, neuronal atrophy in LS and NARP syndromes is not surprising. The remainder of this review will focus on ATP6 and the effects of mutation within the protein this gene encodes.

Mitochondrial Structure and Function

The mitochondrion is a dual-membrane organelle consisting of an acidic inter-membrane space separated from the less acidic matrix by a highly impermeable membrane (Alberts et al., 2004). Embedded in the inner-membrane is the electron transport chain that consists of the cytochrome complexes and the critical ATP synthesizing enzyme ATP synthase. High energy electrons are delivered to the electron transport chain via the activated carrier molecules NADH and/or $FADH_2$ (NADPH in Plants) from the glycolytic and citric acid cycles in the cytoplasm (Alberts et al., 2004). Upon arrival at complex I, the electrons are passed down their energy gradients while the energy that is subsequently released is coupled to pump protons from the matrix into the inter-membrane space. Thus, a proton gradient high in potential energy is established. These protons are then allowed to flow down their concentration gradient through the enzyme ATP synthase back into the matrix. The energy from the flow of protons through ATP synthase is coupled to the un-favorable ADP + P_i $\rightarrow$ ATP reaction. This mechanism for producing ATP is far more efficient than glycolysis alone, yielding over 30 ATP per molecule of glucose compared to 2 ATP from glycolysis (Alberts et al., 2004).

ATP Synthase and ATP6

At the end of the electron transport chain on the inner-mitochondrial membrane is the enzyme ATP synthase, which is solely responsible for all of the ATP produced within the mitochondria. It is important to note that this enzyme is also present in the plasma membranes of bacteria and in the thylakoid membranes of chloroplasts (Noji and Yoshida, 2001). Essentially, ATP synthase couples the energy released from protons flowing down their electrochemical gradients to drive mechanical rotation of the enzyme and synthesize ATP. ATP synthase has a soluble F_1 portion comprised of five different subunits that act as the site of catalysis, and an insoluble F_0 sector embedded in the membrane comprised of ten different subunits (Baracca et al., 2000; Elston et al., 1998). F_1 and F_0 are connected via

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a common rotary shaft (Noji and Yoshida, 2001; Elston et al., 1998; Alberts et al., 2004).

Particularly important is the "a" subunit of F_0 and the 10-14 "c-ring" subunits it is connected to (Noji and Yoshida, 2001). Subunit "a" is encoded by the mitochondrial ATP6 gene and forms the channel for protons to flow through (referred to as ATP6 interchangeably) (Elston et al., 1998; Alberts et al., 2004). While protons flow through ATP6, they drive the rotation of the c-rings and consequently rotate the common shaft linking F_1 and F_0 (Elston et al., 1998; Noji and Yoshida, 2001). This rotational energy is used by the β subunit in F_1 to synthesize ATP from ADP (Noji and Yoshida, 2001; Alberts et al., 2004). Interestingly, this rotary enzyme can operate in reverse (i.e. ATP hydrolysis). The cell carefully controls its supply of ATP, not wanting too little or too much of it. If there is too much ATP, simple Brownian motion of the pool of ATP and ADP will lead to more ATP binding to the β subunit on ATP synthase than ADP. As a result, ATP hydrolysis occurs and drives the rotary shaft linking F_0 and F_1 in the opposite direction (Elston et al., 1998; Alberts et al., 2004). This results in protons being pumped from the matrix into the inter-membrane space and stores the excess energy within the proton gradient rather than ATP (Elston et al., 1998).

Inheritance and Molecular Genetics of ATP6

Only the ATP6 and ATP8 subunits of ATP synthase are encoded in the 16,569 base-pair circular mitochondrial genome located in the matrix, while all other subunits are in the nuclear genome (Schon et al., 2001; Alberts et al., 2004). Consequently, the inheritance pattern for mitochondrial genes is different from that of nuclear genes. The oocyte contains the vast majority of the mitochondria that the embryo will receive, and the specific mitochondria that are packaged into the egg is a random event (Alberts et al., 2004). Within ATP6 of LS and NARP patients there is a T8993G mutation that causes leucine156 (neutral) to become arginine (positively charged) (Holt et al., 1990), or a much less common and studied T8993C mutation (de Vries at al., 1993). Therefore, initial studies of LS and NARP patients tested for maternal inheritance of the mutant ATP6 gene. This mode of inheritance was confirmed by Ciafaloni et al. (1993).

After confirming maternal inheritance, the significant gap in knowledge was determining the dosage of mutant mitochondrial DNA (heteroplasmy) necessary to cause NARP or Leigh syndromes. Because both exhibit distinct clinical and pathological phenotypes but are caused by an identical mutation within the same gene, there must be a dosage necessary to cause disease difference between the two. Tautch et al. performed the first assessment of mutant ATP6 heteroplasmy in a family with NARP and Leigh syndromes (1992). They determined that a LS patient had greater than 95% T8993G ATP6 DNA in her skin fibroblasts, but that her mother who had NARP harbored between 71% and 39% T8993G DNA (Tautch et al., 1992). The LS patient's uncle also had NARP with 78% T8993G, and her aunt died at age 1 with over 95% mutant ATP6 (Tautch et al., 1992). This study indicated that a large percentage of mutant ATP6 was required to cause either NARP or LS.

Subsequent studies conducted on a larger scale, including the one by White et al. (1999) that evaluated T8993G heteroplasmy in 56 pedigrees with LS and/or NARP, set the threshold for NARP at 70% to 90% mutant ATP6 and LS at any dosage above 90% to 95% (Schon et al., 2001). If we return to the Tautch et al. study, it is interesting to note that the NARP mother had variable heteroplasmy of the T8993G mutation throughout her tissues (1992). As detailed in their manuscript, this is likely due to a random asymmetrical distribution of mutant mitochondrial DNA during mitotic cell divisions (Tautch et al., 1992). Perhaps, during development the mutant and wild-type mitochondria randomly separated unequally and the skin fibroblasts received 71% T8993G while the lymphoblasts inherited only 39% of the mutant mitochondrial DNA as in the Tautch et al. study (1992). However, all studies of NARP and LS patients have demonstrated that the afflicted individuals harbor 70% to 90% for NARP and 90% to 95% for LS on average with no significant difference between the T8993G and T8993C mutations (Tautch et al., 1992; White et al., 1999; Schon et al., 2001).

At the molecular genetics level, ATP6 is like all mitochondrial genes in that it is composed of only coding DNA that cannot be alternatively spliced (Albert's et al., 2004). Interestingly, the triplet code for amino acids in the mitochondrial genome is different from that of the nucleus. Therefore, several studies to be discussed will allude to their need to alter the nucleotide sequence of ATP6 in order to dictate the correct amino acid sequence when allotopically expressed in the nucleus of mammalian cells.

Effects of T8993G on ATP Synthesis, and ATP Synthase Structure, Assembly, and Catalytic Activities

It is obvious that ATP6 plays a critical role in ATP synthesis because it forms the channel for proton flow through ATP synthase and regulates the rotation of the c-ring as a result. As stated earlier, LS and NARP syndromes both result from dysfunction of the ATP6 gene, and their pathology is neurodegeneration. Once the heteroplasmic effects of mutant ATP6 were investigated, the next gap in knowledge was whether a decrease in ATP synthesis is correlated with the LS and NARP pathology. Based on the logic that extremely high demand for ATP by the brain is not satisfied by dysfunctional ATP synthase, initial studies attempted to measure ATP production as well as other characteristics of respiration in the cells of LS and NARP patients.

Trounce et al. aimed to determine if lymphoblasts from LS patients had decreased ADP stimulated respiration (state III respiration) by analyzing the respiration rate and ADP phosphoralated to oxygen molecules reduced ratio (ADP/O) (1994). Their results indicated that lymphoblasts harboring between 95% and 100% T8993G DNA had a 26% to 50% decrease in respiration rate, and 30% to 40% decrease in the ADP/O ratio. In addition, if the same lymphoblasts were enucleated and then fused with mitochondrial DNA deficient ρ^0 cells to create a cytoplasmic hybrid (cybrid), the T8993G homoplasmic mutants had a decreased respiration rate and ADP/O ratio similar to

that of the lymphoblasts (Trounce et al., 1994). These results provided a critical demonstration of the ability of the T8993G mutation to reduce ATP synthesis and specifically, indicated a dysfunctional proton channel as the underlying cause.

Though a decrease in ATP synthesis was linked to the T8993G mutation, subsequent study by Houstek et al. further characterized the effects of this mutation on ATP synthase specifically (1995). Using skin fibroblasts containing 99% T8993G DNA, they re-affirmed the Trounce et al. (1994) findings of decreased ATP synthesis but, more importantly, analyzed the effect of the mutation on the assembly of ATP synthase (Houstek et al., 1995). Incorporating mutant cells from the heart, kidney, muscle, and liver into their study, they found very little ATP synthase existing in the normal 620 kDa form and many smaller complexes between 150 kDa and 460 kDa. When immunoprecipitating the F_0 and F_1 subunits individually, the F_0 concentration was significantly reduced in certain tissues (Houstek et al., 1995). These results raise the possibility that the T8993G mutation may be detrimental as a result of it preventing proper ATP synthase complex assembly, rather than altering the properties of the proton channel itself (Houstek et al., 1995).

To fill help fill this gap in knowledge and solve the emerging controversy, Garcia et al. evaluated the effects of T8993G on ATP synthase function and assembly and determined if a reduction in proton flow through ATP6 is the prominent cause of decreased ATP synthesis (2000). When the potential across the inner-mitochondrial membrane was measured in fibroblasts containing either 91% or 100% T8993G DNA, they found that the potential was decreased compared to controls (Garcia et al., 2000). In addition, ATP hydrolysis was similar between mutants and controls, which indicates that the enzyme was assembled (Garcia et al., 2000). The most significant finding was that the potential across the membrane is reduced when T8993G is present, indicating that there is reduction in proton flow through ATP6 (Garcia et al., 2000). Thus, Garcia et al. concluded that the mutation alters the catalytic activity of ATP6 rather then the stability or assembly of the enzyme, as previously thought (2000). This finding contradicts the results of Houstek et al. (1995).

The debate over whether the mutation alters ATP synthase catalysis or assembly/structure led to several studies. First, Baracca et al. published a manuscript supporting Garcia et al. (2000) that directly analyzed the rate of active proton pumping through ATP6 as well as overall ATP synthase activity, ATP hydrolysis, and ATP synthesis in T8993G sub-mitochondrial particles isolated from platelets (Baracca et al., 2000). ATP hydrolysis was unaffected, ATP synthesis was decreased by ~ 20X, whole enzyme function was decreased, but proton pumping was unaffected in mutants (Baracca et al., 2000). Thus, they concluded that the enzyme was assembled with a small structural alteration in T8993G ATP6 that reduced proton flow and ATP synthesis. Interestingly, this and the Garcia et al. (2000) study relied on the logic that if the ATP hydrolysis function is the same as controls, then the enzyme must be properly assembled.

Within a year of the Garcia et al. (2000) and Baracca et al. (2000) studies, Nijtmans et al. conducted research on the assembly of the ATP synthase holoenzyme in osteosarcoma cells transformed with T8993G mitochondria (2001). Using immunoblots to detect the proper assembly of ATP synthase, they showed that the mutant enzyme existed in several different sub-complexes (Nijtmans et al., 2001). This illustrates that the mutant ATP synthase is both improperly assembled and less stable (Nijtmans et al., 2001). Consequently, they concluded that the failure for proper ATP synthase assembly and decreased stability likely contributed to disease pathology, along with a decrease in catalytic function. Thus, the debate arguably ended between the two differing hypotheses because evidence supported the combination of disassembly/decreased stability and catalytic dysfunction as synergistic causes of impaired ATP synthesis (Nijtmans et al., 2001; Baracca et al., 2000; Garcia et al., 2000; Houstek et al., 1995).

Evaluation of ATP6 in *E. coli*

The ATP synthase enzyme in humans exhibits an incredible degree of similarity to that of E. coli (Alberts et al., 2004). Therefore, E. coli serves as an ideal model for studying ATP synthase activity and characteristics, though, mammalian cybrids remain the principal model. The only model of LS and NARP syndrome outside of mammalian cells was created by Hartzog and Cain (2003). They transformed ATP6 deficient E. coli with the leucine156 to arginine substitution that is identical to that of T8993G (Hartzog and Cain, 2003). When culture growth was measured, mutant cells were unable to grow, therefore, indicating the loss of ATP synthesis (Hartzog and Cain, 2003). In addition, when the F_1 portion was removed and functional F_0 remained, the proton conductance through the channel was not distinguishable from negative controls (Hartzog and Cain, 2003). These results mirrored those found in mammalian cells and cybrids, demonstrating the remarkable similarity between human and bacterial ATP synthase.

Altering the Cellular Content of T8993G *In Vivo*

Unlike the static nuclear genotype, the mitochondrial genetic makeup of a tissue or cell culture changes during mitotic divisions due to the assortment and divisions of individual mitochondria. Whether this model could be used as a line of evidence suggesting that an individual with the T8993G mutation may later develop a more wild-type phenotype remained unknown until 1999. Manfredi et al. employed a line of cybrids heteroplasmic for T8993G to examine the changes in mutant dosage over time (1999). They used the mitochondrial ATP synthase inhibitor oligomycin and the respiratory straining galactose carbon source to stress a culture of heteroplasmic cybrids. When the selective media was removed and the cells were allowed to recover, the percentage of T8993G within each cell decreased by 12% (Manfredi et al., 1999). Cybrid divisions were not a rapidly occurring event during the study, indicating that intra-cellular rather than inter-cellular selection for wild-type mtDNA was occurring (Manfredi et al., 1999). The authors attribute this to the T8993G mutation possibly slowing or impairing mitochondrial division/replication.

Over time this would lead to a decrease of T8993G DNA (Manfredi et al., 1999).

If these findings are applied to an LS patient or NARP patient, development and cellular growth could reduce the percentage of mutant DNA. The Manfredi et al. results do not make constant cell division necessary for this to occur because selection occurs intra-cellularly. Thus, the cell simply needs to grow.

Oxidative Stress, Apoptosis, and T8993G ATP6

Oxidative stress resulting from the generation of reactive oxygen species (ROS) in dysfunctional mitochondria or in response to environmental toxins can produce a variety of detrimental effects or apoptosis (Alberts et al., 2004). Because T8993G has such profound effects on ATP synthesis and leads to cell death if heteroplasmically high, researchers believed it was linked to oxidative stress and that may be the ultimate cause of neuronal atrophy (Geromel et al., 2001; Mattiazzi et al., 2004).

Using cultures of skin fibroblasts, Geromel et al. aimed to determine if cells from ATP synthase deficient patients with >90% T8993G were dieing from apoptosis (2001). Cells were stained with the general apoptotic marker annexin V, and then with TUNEL to reveal the presence of fragmented DNA (Geromel et al., 2001). Many previous studies have linked the overproduction of superoxides to causing apoptosis thus, they tested and found an elevated presence of the superoxide dismutase enzymes (SODs) that scavenge the free radicals (Geromel et al., 2001). In order to link ROS accumulation/oxidative stress to instigating apoptosis the mutant fibroblasts were treated with TAPBN, which reduces free radicals (i.e. an antioxidant), and then stained with TUNEL (Geromel et al., 2001). Interestingly, elevated SOD expression and TAPBN rescued the cells from apoptosis (Geromel et al., 2001). These experiments provided the first evidence indicating that oxidative stress is involved in triggering apoptosis in mutant ATP6 cells and that they do not die simply from running out of energy (Geromel et al., 2001).

As a result of anti-oxidant treatment rescuing mutant ATP6 cells from apoptosis, Mattiazzi et al. examined the effects of anti-oxidants on ATP synthesis (2004). They created cybrids formed by the fusion of mtDNA deficient osteosarcoma cells with enucleated platelet cells from NARP or LS individuals (Mattiazzi et al., 2004). It was found that those cells produced elevated levels of ROS that damaged mitochondrial lipids and increased levels of superoxide dismutases (Mattiazzi et al., 2004). The T8993G mutation was also found to cause dysfunction in non-mutated complexes of the electron-transport chain, further contributing to decreased ATP synthesis (Mattiazzi et al., 2004).

Remarkably, treatment of mutant cybrids with NAC and DHLPA (antioxidants) reduced the amount of ROS and increased ATP synthesis (Mattiazzi et al., 2004). It is feasible that this finding implicates ROS species as electron-transport chain disruptors because removing them restores some ATP synthesis (Mattiazzi et al., 2004). Lastly, in support of the decreased proton flow through ATP6 hypothesis they found an increased matrix pH in mutants as would be expected if protons were not flowing into it (Mattiazzi et al., 2004).

Effects of Mutant ATP6 on Tumor Growth

In the vast majority of cancer cells, there is some mutation within the mitochondrial genome (Shidara et al., 2005). Based on this knowledge, Shidara et al. observed the role of T8993G ATP6 in tumor cell growth in mice (2005). HeLa cells (immortal cell line created from cervical cancer) were fused with enucleated T8993G cells from patients with NARP to form cybrids (Shidara et al., 2005). These HeLa cybrids were transplanted into mice and tumor growth was compared with mice receiving non-cybrid HeLa cells (Shidara et al., 2005). Surprisingly, mutant ATP6 led to faster tumor growth and decreased apoptosis compared to WT ATP6 (Shidara et al., 2005).

As discussed previously, the T8993G leads to an increase in ROS production, reduced ATP synthesis, and apoptosis, yet why is it that cancerous cells often have dysfunctional mitochondria or even lost ATP synthase function as in renal carcinomas (Mattiazzi et al., 2004; Shidara et al., 2005; Simonnet et al., 2002)? Shidara et al. attribute this discrepancy with the Mattiazzi et al. study as the result of physiological differences between fibroblasts and HeLa cells (2005). HeLa cells satisfy their energy requirements using the glycolytic reactions more than oxidative-phosphoralation in contrast to fibroblasts that perform the opposite (Shidara et al., 2005). However, this finding is significant because if a cell becomes cancerous it is possible that the T8993G mutation will exacerbate tumor growth.

Therapeutic Intervention

Currently, there are no truly effective therapies for LS and NARP syndromes. However, from the aforementioned research a novel possibility for therapeutic intervention arises; the introduction of antioxidants and/or over expression of SOD enzymes (Geromel et al., 2001; Mattiazzi et al., 2004). If patients were able to absorb significant quantities of antioxidants into their cells, it may alleviate some of their symptoms and be especially beneficial to NARP patients. For LS patients to survive gene therapy intervention is needed because they die from the disease in infancy.

A study by Manfredi et al. may have revealed the key to treating the disease (2002). Their research team converted the ATP6 codons into those compatible with the differing universal genetic code of the nucleus for amino acids and attached a mitochondrial targeting sequence (Manfredi et al., 2002). This gene constructed of WT ATP6 was cloned into a vector and either transfected chemically or through a modified adeno-associated virus (AAV) into the nucleus of human HEK (human embryonic kidney) cells homoplasmic for T8993G ATP6 mitochondrial DNA (Manfredi et al., 2002). They found that the WT ATP6 being transcribed in the nucleus was trafficked to the mitochondria and incorporated into ATP synthase (Manfredi et al., 2002). These cells demonstrated a significant increase in ATP synthesis as a result of allotopically expressed WT ATP6 (Manfredi et al.,

2002). Thus, this profound finding may lead to the expression of WT ATP6 in LS and NARP patients (Manfredi et al., 2002). Perhaps, since the disease can be prenatally diagnosed, transfection of developing cells will lead to increased distribution of the functional gene. However, it is important to note that the mutant form of ATP6 will still be expressed to some degree.

Future Research

A possibility for future research includes further examination of the effect of T8993G on oxidative stress, and if ROS accumulation decreases ATP synthesis more than the defective proton channel alone. This will allow pharmaceutical companies to know how to direct their short-term research efforts to design the first therapeutics for LS and NARP syndromes. A second possibility is continuing research on the effectiveness of nuclear transfection of LS and NARP patient cells with WT ATP6 using an adeno virus. Because cells can tolerate a significant degree of mutant ATP6, as illustrated by the 90% T8993G threshold for LS, allowing cells to produce a few percent more of WT ATP6 may bring them back below threshold and eliminate symptoms altogether.

Conclusion

The debilitating or fatal conditions caused by mutation within the ATP6 subunit of ATP synthase are all the reason to continue research within the field of mitochondrial genetics. This review has presented three major hypotheses that attempt to fill the central gap in knowledge, which is how the alteration of ATP6 reduces ATP synthesis and causes disease. First, ATP synthesis is decreased as a result of the failure of proper ATP synthase assembly and stability. Second, the substitution of the charged amino acid arginine for neutral leucine causes the proton channel ATP6 forms to resist or prevent proton flow thereby decreasing c-ring rotation and ATP synthesis. Third, the T8993G mutation and its effects on ATP synthase result in the accumulation of ROS causing oxidative stress that induces apoptosis and reduces electron-transport chain function. All three of these hypotheses are currently valid and widely accepted within the field, and it is likely that all are involved in disease pathogenesis. Thus, the new model combines aspects of all these hypotheses and their supporting evidence to show that all are intrinsically related.

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