Mitochondria, NO and neurodegeneration

M. Flint Beal

Neurochemistry Laboratory, Neurology Service/WRN 408, Massachusetts General Hospital and Harvard Medical School, 32 Fruit Street, Boston, MA 02114, U.S.A.

Abstract

A role for mitochondrial dysfunction in neurodegenerative disease is gaining increasing support. Mitochondrial dysfunction may be linked to neurodegenerative diseases through a variety of different pathways, including free-radical generation, impaired calcium buffering and the mitochondrial permeability transition. This can lead to both apoptotic and necrotic cell death. Recent evidence has shown that there is a mitochondrial defect in Friedreich’s ataxia, which leads to increased mitochondrial iron content, that appears to be linked to increased free-radical generation. There is evidence that the point mutations in superoxide dismutase which are associated with amyotrophic lateral sclerosis may contribute to mitochondrial dysfunction. There is also evidence for bioenergetic defects in Huntington’s disease. Studies of cybrid cell lines have implicated mitochondrial defects in both Parkinson’s disease and Alzheimer’s disease. If mitochondrial dysfunction plays a role in neurodegenerative diseases then therapeutic strategies such as coenzyme Q10 and creatine may be useful in attempting to slow the disease process.

Introduction

A potential critical role of mitochondrial dysfunction in neurodegenerative diseases is becoming increasingly compelling. Mitochondrial dysfunction leads to a number of deleterious consequences for the cell, including impaired calcium buffering, generation of free radicals, activation of nitric oxide synthase, activation of the mitochondrial permeability transition and secondary excitotoxicity. This can lead to both apoptotic and necrotic cell death, depending on the severity of the insult. Neurodegenerative diseases have widely disparate genetic aetiologies but may share mitochondrial dysfunction as a final common pathway. Recent evidence has implicated a mitochondrial defect in Friedreich’s ataxia, since the protein frataxin is a nuclear-encoded mitochond-
**Alzheimer's disease**

Alzheimer's disease is the most common of the neurodegenerative diseases. The most important risk factor is advancing age [1]. There is also epidemiological data showing that head injury is a risk factor and that ingestion of either oestrogens or anti-inflammatory medications reduces one's risk. The illness occurs in both familial form, which is autosomal-dominant inherited, and as an apparently sporadic illness. The autosomal-dominant inherited form is associated with mutations in the amyloid precursor protein, as well as two novel proteins entitled presenilin-1 and presenilin-2. Familial Alzheimer's disease accounts for approximately 5% of all cases. The remaining sporadic cases of Alzheimer's disease show an increased risk in families of 2.5–3-fold as well as some evidence for maternal inheritance. The latter studies have shown an increase in the female-to-male ratio in the parental generation of Alzheimer's disease probands [2,3]. In a group of families in which there was both an affected parent as well as at least two affected siblings the ratio of affected mothers to fathers in the parental generation was 9:1 [3].

A number of studies have implicated metabolic defects in Alzheimer's disease. There is a substantial body of work utilizing positron emission tomography, which consistently shows reduced glucose metabolism in the temporoparietal regions of Alzheimer's disease patients. This occurs quite early in the disease course [4]. This has also been demonstrated in patients at risk for Alzheimer's disease as well as in asymptomatic patients who are homozygous for the Apo e4 allele, a known risk factor for sporadic Alzheimer's disease [5,6]. The positron emission tomography studies also show increased oxidative utilization in comparison with glucose utilization in Alzheimer's disease patients, and the latter observation has been confirmed with direct measurements in arterial and jugular venous samples [7,8]. An uncoupling of glucose metabolism was previously demonstrated in brain-biopsy specimens [9]. Phosphorous magnetic resonance spectroscopy has demonstrated abnormalities in either phosphocreatine or inorganic phosphate in Alzheimer's disease patients as compared with elderly controls [10–12].

The most consistent defect in mitochondrial electron-transport enzymes has been a deficiency in cytochrome oxidase activity. This was initially repor-
ted in Alzheimer’s disease platelets [13]. Subsequent work has demonstrated that there is reduced cytochrome oxidase activity in post-mortem brain tissue from Alzheimer’s disease patients [13–15]. The cytochrome oxidase activity showed a reduction in catalytic activity and normal amounts of cytochromes \( a + a_3 \), suggesting that reduced complex-IV activity is a consequence of abnormal catalytic activity rather than decreased enzyme levels.

An innovative technique to study mitochondrial defects in Alzheimer’s disease has been the application of cybrid technology, which was pioneered by King and Attardi [16]. This technique involves the transfer of mitochondria from living patients to cell lines which are deficient in mitochondria (Rho cells). The cell lines are initially depleted of mitochondrial DNA by exposing them to low concentrations of ethidium bromide, which preferentially inhibits mitochondrial DNA replication. The exposed cells lose their mitochondrial DNA and assume an anaerobic phenotype. Recent studies using the cybrid technique demonstrated that cytochrome oxidase defects found in Alzheimer’s disease platelets could indeed be transferred into Rho cells to form cybrids which also demonstrated the cytochrome oxidase defect [17,18]. Furthermore, the ensuing cybrid cell lines showed markedly increased free-radical production. These cell lines also showed impaired intracellular calcium buffering [19]. The cybrids showed elevated basal cytosolic calcium concentrations as well as enhanced sensitivity to inositol 1,4,5-trisphosphate-mediated calcium release. These findings are consistent with prior observations in Alzheimer’s disease fibroblasts [20,21]. They are also consistent with the finding of decreased calcium uptake in mitochondria from Alzheimer’s disease fibroblasts [22].

These findings suggest that there may be a mitochondrial-DNA-encoded defect accounting for reduced cytochrome oxidase activity in Alzheimer’s disease. Initial attempts to find point mutations in mitochondrial DNA were hampered by the presence of cytochrome oxidase subunits in the nuclear genome which occur as nuclear pseudogenes [23,24]. These are mitochondrial DNA sequences that were randomly incorporated into the nuclear genome very early in evolution. An initial report of point mutations in cytochrome oxidase-1 and cytochrome oxidase-2 mitochondrial-DNA-encoded subunits was later found to be due to the presence of polymorphisms in a large nuclear pseudogene [18].

A consequence of mitochondrial dysfunction may be increased free-radical production. Consistent with this we found that mitochondrial DNA showed a three-fold increase in concentrations of 8-hydroxy-2-deoxyguanosine, a marker of oxidative damage to DNA [25]. Other studies have consistently shown increased tissue concentrations of markers of lipid peroxidation as well as protein carbonyl groups [26]. Novel spin-trapping techniques also demonstrated increased oxidative damage to both lipids and proteins [27]. Immunocytochemical studies have provided evidence for oxidative damage at the cellular level [28–32]. A recent study showed that there appeared to be increased amounts of redox-active iron within neurofibrillary-tangle-bearing neurons [31]. Two studies have demonstrated that neurofibrillary-tangle-bearing neurons show increased immunostaining with antibodies to 3-nitrotyrosine, a marker for peroxynitrite-mediated damage [31,32].
Friedreich's ataxia

The role of mitochondrial dysfunction in Friedreich's ataxia has been greatly strengthened by recent findings that the protein which encodes the genetic defect is localized to mitochondria. Friedreich's ataxia is characterized by neurodegeneration involving the spinocerebellar pathways as well as a cardiomyopathy. The gene product has been designated frataxin. It is typically caused by an expansion of a polymorphic GAA trinucleotide repeat situated in the first intron of the corresponding gene [33]. Typically, there is an extremely large expansion of GAA in one allele and a lesser expansion in the opposite allele. The age of onset varies inversely with the length of the GAA-repeat increase in the allele containing the smaller GAA expansion [34]. It has been demonstrated that the GAA increase results in an abnormal DNA structure as well as an impairment of transcription, resulting in reduced steady-state levels of mature frataxin mRNA [35,36]. There is a correlation between regions of degeneration observed in the disease and the sites of frataxin transcription, which are highest in the heart, spinal cord and dorsal root ganglia.

Studies in yeast have shown that there is a gene homologous to the human frataxin protein [37–39]. This gene encodes a mitochondrial protein involved in iron homoeostasis and respiratory function [39]. Both antibody staining and studies of human frataxin linked to green fluorescent protein have shown that the frataxin protein is localized to mitochondria [38–40]. Disruption of the yeast homologous protein results in respiratory insufficiency with an inability to carry out oxidative phosphorylation and loss of mitochondrial DNA [37,39]. Yeast with disruption of the frataxin homologue showed markedly increased iron transport, increases in iron content and hypersensitivity to oxidative stress mediated by hydrogen peroxide [39].

These observations suggest that impaired function of this protein most likely leads to mitochondrial dysfunction as well as hypersensitivity to oxidative stress, which may be mediated by iron-catalysed Fenton chemistry. Recent studies in patients have supported this supposition. Endomyocardial biopsies of two patients with Friedreich's ataxia showed marked deficiencies of aconitase and complexes I and II–III of the electron-transport chains [41]. These enzymes contain iron–sulphur clusters and are known to be particularly susceptible to oxidative stress. Other evidence implicating oxidative stress in Friedreich's ataxia is the finding that patients with abnormalities in vitamin E transport protein can show a similar phenotype [42–44].

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a prototypical neurodegenerative disease of later life characterized by progressive muscle weakness, atrophy and spasticity. Approximately 10% of cases show familial autosomal-dominant inheritance and a major advance in understanding ALS was the identification of point mutations in the enzyme superoxide dismutase in approximately 25% of patients who have familial ALS [45]. More than 60 mutations have now been
associated with the disease. These mutations typically affect the protein back-
bone of the enzyme and may interfere with normal dimer interactions [46].

The observation that mutations in superoxide dismutase cause familial
ALS suggested that oxidative injury might be playing a role in pathogenesis.
Several observations argue that the mutations result in a gain of an adverse
property of the mutant SOD1 protein. These include the dominant inheritance
pattern in familial ALS, the lack of correlation between enzyme activity and
disease manifestations, the absence of motor neuron disease in mice with a
knockout of SOD1 and the observation that overexpression of the mutant
enzyme in transgenic mice (which have increased superoxide dismutase activ-
ity) leads to motor-neuron degeneration [47–49].

With rare exceptions, mutations occur outside the active site of the
enzyme and they also alter zinc binding, which may further destabilize the pro-
tein backbone [50,51]. These changes may relax the conformation of the active
channel and thereby allow increased access of hydrogen peroxide or peroxyni-
trite to the active site copper, which is predicted to increase generation of
hydroxyl radicals and/or nitronium ions, which can nitrate proteins [52,53].
Increased production of hydroxyl radicals by mutant SOD1 has been shown in
vitro and we found increased 3-nitrotyrosine levels in other markers of oxida-
tive injury in two different strains of transgenic ALS mice in vivo [52,54,55].
Mitochondria are particularly vulnerable to oxidative stress and mitochondrial
swelling and vacuolization are among the earliest pathological features of two
strains of transgenic ALS mice with SOD1 mutations [47,49]. Mice with the
G93A human SOD1 mutation showed altered electron-transport enzymes, and
expression of the mutant enzyme in vitro resulted in a loss of mitochondrial
membrane potential as well as elevated cytosolic calcium concentrations
[56,57].

In sporadic ALS there is also evidence that suggests impairment of energy
metabolism [58]. Reduced glucose metabolism has been observed in the cere-
bral cortex of sporadic ALS patients using positron emission tomography [59].
In post-mortem tissue we did not find alterations in electron-transport
enzymes; however, this could be due to heterogeneity of defects among
patients, which would obscure them due to an averaging effect [57]. A recent
study of muscle-biopsy specimens showed a reduction in complex-I activity
[60]. Muscle biopsies have also demonstrated an accumulation of mitochondria
and proximal axons, increased mitochondrial volume and calcium levels
[61,62]. Studies of peripheral blood lymphocytes from sporadic ALS patients
show increased cytosolic calcium in impaired responses to uncouplers of
oxidative phosphorylation [63]. A recent study using the cybrid cell technique
suggested that there were mild decreases in complexes I and IV activities associ-
ated with sporadic ALS [64]. Liver biopsies have shown enlarged mitochondria
with intramitochondrial inclusions in sporadic ALS [65]. Abnormal mitochon-
dria with protrusions were observed in anterior horn cells of a familial ALS
patient who was later determined to have a superoxide dismutase mutation
[66]. There is substantial recent evidence demonstrating oxidative damage in
both familial and sporadic ALS patients [67,68]. We and others have observed
increased protein carbonyl groups in the motor cortex and spinal cord of ALS
patients. We recently found increased concentrations of 3-nitrotyrosine and its major metabolite 3-nitro-4-hydroxyphenyl acetic acid in the thoracic and lumbar spinal-cord tissue of both sporadic and familial ALS patients with SOD1 mutations [69]. Immunocytochemical staining also demonstrated increased 3-nitrotyrosine staining in anterior horn cells. We also found increased levels of 8-hydroxy-2-deoxyguanosine in the motor cortex of sporadic ALS patients [54].

**HD**

The genetic defect in HD consists of an expanded CAG repeat in a gene located on chromosome 4. The gene encodes a novel protein, designated huntingtin, which is widely distributed in both the peripheral tissues of the body as well as in the central nervous system. The means by which it leads to neuronal degeneration is, as yet, obscure. A number of potential interactions have been considered. These include impairment of transcription, cross-linking to other proteins via transglutaminase, direct interactions with other proteins and abnormal aggregation leading to intracellular inclusion bodies [70]. It was recently demonstrated that cleavage of proteins containing expanded polyglutamine stretches in the pathological range found with HD leads to spontaneous aggregation \textit{in vitro} [71]. The aggregates at the electron-microscopic level appear to have a structure similar to aggregations that have been recently found in both HD patients as well as in a transgenic mouse model of HD [18,70]. In the sporadic HD patients and transgenic mice there is evidence for the development of intranuclear inclusion bodies, which stain with N-terminal antibodies to huntingtin as well as with antibodies to ubiquitin [70]. They do not appear to contain other proteins. These intranuclear inclusion bodies are more prevalent in patients with juvenile-onset HD. In a transgenic mouse model of HD they occur early in the disease course [18]. Whether they are directly linked to the pathogenesis of the neurodegeneration, however, is as yet unclear.

There is a substantial body of evidence implicating defects in energy metabolism in HD. Decreased glucose metabolism has been demonstrated using positron emission tomography. This occurs in presymptomatic patients. We utilized proton magnetic resonance spectroscopy to demonstrate that there are elevated lactate levels in both the occipital cortex and the basal ganglia of HD patients [72,73]. Increased lactate concentrations occur in the basal ganglia of some but not all presymptomatic patients, suggesting that metabolic defects can precede clinical manifestations of the illness. Other authors found increased lactate in both the frontal cortex as well as in the occipital cortex and cerebellum [74,75]. We observed increased lactate concentrations in both the parietal and supplementary motor cortices, suggesting that there is a widespread metabolic defect in HD brain tissue. Furthermore, we found an increase in the phosphocreatine-to-inorganic phosphate ratio in resting gastrocnemius muscle of HD patients, providing evidence that there are defects in energy metabolism in peripheral tissues as well [76]. The latter observation is consistent with reports of progressive weight loss in HD patients despite increased caloric intake [77].
In post-mortem brain tissue the most consistent defect which we and others have found is a decrease in complex-II–III activity in the basal ganglia [78,79]. Smaller decreases in cytochrome oxidase were less consistent. Complex-I and citrate synthase activity showed no changes. The finding of decreased complex-II–III activity is of interest since genetic defects in complex-II have been associated with basal ganglia degeneration [80]. Comparable defects in experimental animals result in striatal degeneration [81]. Recent studies in cultured fibroblasts from HD and control patients also showed evidence of mitochondrial dysfunction [82]. Ionomycin-induced calcium influxes resulted in depolarizations of the mitochondrial membrane potential that were much more marked in HD fibroblasts compared with normal controls.

One proposed mechanism by which the HD gene defect could lead to impaired energy metabolism is by an interaction between huntingtin and glyceraldehyde 3-phosphate dehydrogenase, a critical component of the glycolytic pathway [83]. It was suggested that an increase in polyglutamine repeats in huntingtin potentially inhibits the enzyme. It has also been suggested that it may be possible to cross-link huntingtin to glyceraldehyde 3-phosphate dehydrogenase via transglutaminase [84]. This could also lead to inactivation of the enzyme. We found that intrastriatal administration of the glyceraldehyde 3-phosphate dehydrogenase inhibitor iodoacetate produces dose-dependent striatal lesions which correlate with inhibition of enzyme activity [85]. However, we were unable to confirm a decrease in glyceraldehyde 3-phosphate dehydrogenase activity in post-mortem brain tissue in HD patients [79]. Nevertheless, a competitive inhibition could still occur.

Other evidence implicating mitochondrial dysfunction in the pathogenesis of HD comes from animal models. We initially utilized mitochondrial toxins such as 3-nitropropionic acid, an irreversible inhibitor of succinate dehydrogenase, in an attempt to model HD in both rodents and primates [86,87]. Systemic administration of 3-nitropropionic acid to non-human primates results in both the movement disorder as well as frontal-type cognitive deficits which are similar to those that occur in HD [86]. Histological evaluation showed basal ganglia degeneration with sparing of NADPH-diaphorase interneurons, dendritic abnormalities in spiny neurons and sparing of the nucleus accumbens, all of which are characteristic features of HD neuropathology. The pathogenesis of the lesions as studied in rodents involves the generation of free radicals and nitric oxide [88]. We found that both free-radical scavengers as well as inhibitors of neuronal nitric oxide synthase are highly effective in blocking the development of the lesions.

A major advance for the study of HD comes from the development of a transgenic animal model [89]. Transgenic mice were made to express a large number of CAG repeats in exon 1 of the HD gene. These animals develop normally for about 8 weeks and then develop a movement disorder with tremors and choreiform movements. A characteristic feature of these mice is progressive weight loss despite increased caloric intake. These findings are similar to those observed in HD patients. They therefore suggest that there may indeed be a metabolic defect in these transgenic mice. Our initial studies in
these mice have shown that there is a small elevation of lactate and a reduction in N-acetyl aspartate, as determined by magnetic resonance spectroscopy.

**Therapeutic strategies**

If a defect in energy metabolism underlies the pathogenesis of neurodegenerative disease then a reasonable therapeutic strategy is to utilize compounds which improve mitochondrial dysfunction. We have demonstrated that coenzyme Q₁₀ produces dose-dependent protection against striatal lesions produced by the succinate dehydrogenase inhibitor malonate [90]. We also recently found that it produces marked neuroprotection against 3-nitropropionic acid toxicity and improves survival in a transgenic animal model of ALS associated with point mutations in superoxide dismutase [91]. Coenzyme Q₁₀ also protects against malonate-induced ATP depletions [90]. We found that it produced a significant (36%) decrease in occipital cortex lactate concentrations in HD patients as assessed by magnetic resonance spectroscopy [76]. This has led to the design of a clinical trial to study the effects of coenzyme Q₁₀ with or without remacemide, an N-methyl-D-aspartate antagonist, in the treatment of HD patients.

Another novel therapeutic strategy to ameliorate mitochondrially induced dysfunction is to attempt to buffer intracellular energy stores. We studied the effects of creatine on lesions produced by 3-nitropropionic acid and malonate [92]. Creatine is metabolized by creatine kinase to generate phosphocreatine. Phosphocreatine then serves to buffer ATP concentrations in the cell. Creatine also stabilizes the mitochondrial transition pore [93]. Its importance to brain function is supported by in vivo ³¹P, a magnetic-resonance transfer measurement showing a correlation of creatine kinase flux with brain activity measured by the electroencephalogram, as well as brain 2-deoxyglucose uptake [94].

We found that administration of creatine in the diet produced significant protection against both malonate and 3-nitropropionic acid-induced toxicity [92]. Creatine increased brain levels of phosphocreatine and ATP and protected against 3-nitropropionic-induced lesions. Creatine also protected against 3-nitropropionic acid-induced increases in striatal lactate concentrations, as assessed by proton magnetic resonance spectroscopy. It protected against 3-nitropropionic acid-induced increases in 3-nitrotyrosine. These findings suggest that creatine might be a useful treatment for neurodegenerative diseases.

The secretarial assistance of Sharon Melanson is gratefully acknowledged. This work was supported by NIH grants NS16367, NS31579, AG11337 and AG12992, the Muscular Dystrophy Association and the ALS Association.

**References**