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Superoxide dismutase 1 mutation in a cellular model of amyotrophic lateral sclerosis shifts energy generation from oxidative phosphorylation to glycolysis

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder involving the progressive degeneration of motor neurons in the brain and spinal cord. Mitochondrial dysfunction plays a key role in ALS disease progression and has been observed in several ALS cellular and animal models. Here, we show that fibroblasts isolated from ALS cases with a Cu/Zn superoxide dismutase (SOD1) I113T mutation recapitulate these mitochondrial defects. Using a novel technique, which measures mitochondrial respiration and glycolytic flux simultaneously in living cells, we have shown that SOD1 mutation causes a reduction in mitochondrial respiration and an increase in glycolytic flux. This causes a reduction in adenosine triphosphate produced by oxidative phosphorylation and an increase in adenosine triphosphate produced by glycolysis. Switching the energy source from glucose to galactose caused uncoupling of mitochondria with increased proton leak in $SOD1^{I113T}$ fibroblasts. Assessment of the contribution of fatty acid oxidation to total respiration, suggested that fatty acid oxidation is reduced in SOD1 ALS fibroblasts, an effect which can be mimicked by starving the control cells of glucose. These results highlight the importance of understanding the interplay between the major metabolic pathways, which has the potential to lead to strategies to correct the metabolic dysregulation observed in ALS cases.

1. Introduction

Amyotrophic lateral sclerosis (ALS) or motor neuron disease is an adult onset neurodegenerative disorder characterized by progressive degeneration of motor neurons in the motor cortex, brain stem, and spinal cord. This results in denervation, which is destructive for the muscle leading to progressive weakness and atrophy. Disease progression is rapid, with typical survival only 2–3 years post-diagnosis. The leading therapeutic agent riluzole, offers only a modest extension of life expectancy by approximately 3 months (Lacomblez et al., 1996a, 1996b) and to date there is no known neuroprotective therapy of major impact for this devastating disease.

Approximately 5%–10% of ALS cases have a genetic cause and are termed familial ALS (FALS). Mutations have been identified in approximately 70% of FALS cases with mutations in *C9ORF72*, *TARDBP*, *FUS*, and *SOD1* underlying the most common genetic subtypes (DeJesus-Hernandez et al., 2011; Kwiatkowski et al., 2009; Renton et al., 2011; Rosen, 1993; Vance et al., 2009). Sporadic ALS (SALS) accounts for 90%–95% of all cases with the cause of disease being unknown. However, genome wide association studies have linked several loci such as *ELP3*, *UNC13A*, and *C9ORF72* with increased susceptibility to sporadic ALS (DeJesus-Hernandez et al., 2011; Renton et al., 2011; Simpson et al., 2009; van Es et al., 2009). Expanded GGGGCC repeats in C9ORF72 have been shown to account for 40%–50% of FALS and approximately 7% of SALS cases (Cooper-Knock et al., 2012; Majounie et al., 2012). However, the functional impact of the expanded repeat and the function of the C9ORF72 protein in health and disease have yet to be determined. Although SALS and FALS are clinically indistinguishable, there is a great deal of evidence to suggest that they share multiple pathophysiological mechanisms including protein aggregation, excitotoxicity, axonal transport defects, dysregulation of RNA metabolism, mitochondrial dysfunction, metabolic dysregulation, and oxidative stress (Dupuis et al., 2011; Ferraiuolo et al., 2011).

Mutation of Cu/Zn superoxide dismutase (*SOD1*) is responsible for around 20% of FALS cases (Rosen, 1993). It is a generally accepted

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that toxicity occurs through a "gain of function" and current evidence indicates that mutant SOD1 causes the development of a cascade of interlinked pathophysiological processes affecting motor neurons and glial cells to cause neuronal cell death (Ferraiuolo et al., 2011).

Metabolic dysfunction plays an important role in ALS disease progression. Mutant SOD1 (mSOD1) aggregation in the mitochondria leads to increased free radicals, electron transport chain (ETC), disruption and loss of the mitochondrial membrane potential, which result in mitochondrial morphologic defects and decreased adenosine triphosphate (ATP) production (Borthwick et al., 1999; Bowling et al., 1993; Carri et al., 1997; De Vos et al., 2007; Fujita et al., 1996; Mattiazzi et al., 2002; Menzies et al., 2002; Shaw et al., 1995). This can lead to apoptosis through the release of cytochrome c from the mitochondria. Previous work from our laboratory has shown metabolic gene expression changes in response to stress in both motor neurons isolated from SOD1G93A mice (Ferraiuolo et al., 2007) and fibroblasts from SALS cases (unpublished data).

In healthy individuals, energy intake is in balance with energy expenditure and there is evidence that this balance is disrupted in ALS cases. Individuals with ALS may suffer weight loss and malnutrition because of dysphagia, which leads to decreased energy stores (Dupuis et al., 2011). In addition, most of the ALS patients have higher energy expenditure than healthy subjects (Desport et al., 2001, 2005; Funalot et al., 2009). The biochemical basis for this metabolic dysregulation is not yet clear, but it appears that muscle hypermetabolism and energy deficit are intrinsic to ALS pathogenesis. Furthermore, it has been reported that individuals with ALS have a propensity for glucose intolerance and patients with type 2 diabetes and ALS may have delayed disease onset (Jawaid et al., 2010; Pradat et al., 2010), suggesting that altered metabolism may exert a beneficial effect on the viability of motor neurons.

Metabolic dysregulation and mitochondrial dysfunction are also observed in SOD1 and TDP-43 transgenic mouse models of ALS (Chiang et al., 2010; Dupuis et al., 2004; Fergani et al., 2007; Shan et al., 2010; Xu et al., 2010), with the "hypermetabolism" observed in ALS cases also observed in the mutant SOD1 transgenic mouse. Mutant SOD1 transgenic mice fed on a high fat diet had delayed disease onset, improved motor neuron survival and life span increased by 20% (Dupuis et al., 2004). Why this is the case and the interplay between the key metabolic pathways in ALS is yet to be determined and more detailed understanding of the metabolic dysregualtion may be crucial in developing effective strategies for neuroprotection and metabolic biomarkers of disease.

Easily accessible tissues such as peripheral blood and fibroblasts have been observed to recapitulate pathophysiological abnormalities observed in the central nervous system (CNS) in ALS. Abnormalities such as response to oxidative stress, energy metabolism, and mitochondrial dysfunction have been demonstrated in fibroblast cells cultured from individuals with Alzheimer's disease, Parkinson's disease, and ALS (Mead et al., 2013; Mortiboys et al., 2010; Ramamoorthy et al., 2012). The ease of obtaining and cell culturing of skin fibroblasts make them a valuable model system for the initial study of pathophysiological processes relevant to neurodegeneration. Recently, is has been shown that mutations in the valosin containing protein lead to a loss of the mitochondrial membrane potential, a decrease in the NADH redox index and a loss of ATP production in ALS patient fibroblasts (Bartolome et al., 2013).

In the present study, individuals with an I113T *SOD1* mutation (mSOD1) had skin biopsies taken and fibroblasts were subsequently isolated and cultured. Metabolic pathway analysis was performed using a Seahorse XF24 metabolic analyzer, which allows real-time, physiologically relevant metabolic functional analysis to be performed on living cells. It was found that fibroblasts from mutant SOD1 ALS cases showed significant mitochondrial and metabolic pathway defects compared with age and sex matched controls.

2. Methods

All chemicals were from Sigma, Dorset, UK unless stated otherwise.

2.1. Human fibroblast biosamples

Experiments were carried out using fibroblasts obtained from 3× $SOD1^{I113T}$ patients (all unrelated males from different families) and age- and sex-matched controls. The average age at time of skin biopsy in SOD1 ALS cases and controls was 55.33 years (range 44–62 years), and 53.66 years (range 38–63 years), respectively.

2.2. Fibroblast cultures

Skin biopsies were obtained from the forearm of subjects under sterile conditions after informed consent, in accordance with guidelines set by the local ethics committee. Fibroblast cell cultures were established at the Metabolic Biochemistry and Tissue Culture Unit of the Sheffield Children's NHS Foundation Trust. Monolayers of primary fibroblast cell cultures were routinely maintained in T75 flasks with fibroblast cell culture medium supplied by PAA laboratories, Somerset, UK (FCCM-PAA minimal essential media) supplemented with 10% fetal calf serum (FCS) gold (PAA), 2 mM glutamine (Lonza, Berkshire, UK), 50 μg/mL uridine, vitamins (Lonza 1/100 dilution), amino acids (Lonza 1/100 dilution), 1 mM sodium pyruvate (Lonza), and 1U/mL penicillin/1 μg/mL streptomycin (Lonza) in humid incubators at 37 °C supplemented with 5% CO_2 unless stated otherwise.

2.3. SOD1 western analysis

Fibroblasts were allowed to reach 80% confluency before harvesting by treating with 1x trypsin (Lonza) for 2 minutes, quenching with FCCM-PAA media and collecting the cell pellet by centrifugation (400g, 4 minutes). The pellets were subsequently washed with PBS (Sigma) and the centrifugation process was repeated. The cell pellets were then resuspended in 75 μL of ice-cold buffer A (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES pH 7.4]), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 250 mM sucrose, with protease inhibitor cocktail (Roche, Hertfordshire, UK) added to 10 mL of buffer A and were homogenized using a Kontes mini-homogenizer (Anachem, Bedfordshire, UK). The resultant homogenate was centrifuged at 3000 *g* for 6 minutes at 4 °C. The supernatant was retained on ice, while the pellet was treated again as mentioned previously. The resultant second supernatant was pooled with the first supernatant fraction. The combined supernatants were sonicated on ice at 6 μm using 10 short pulses from a probe sonicator (Soniprep 150, Sanyo, Japan) and then centrifuged at 3000 *g* for 6 minutes at 4 °C. The protein concentration of the cell lysates was determined by a Bradford assay as per the manufacturer's instructions (Pierce, supplied by Thermo Fisher Scientific, Leicestershire, UK). Protein (20 μg) was loaded per lane for western blotting analysis. Samples were denatured in 1× laemmli buffer with 5% (wt/vol) β-mercaptoethanol (Sigma) at 100 °C for 5 minutes. Protein electrophoresis was performed using a Biorad Mini-PROTEAN system (Biorad, Hertfordshire, UK). Resolved proteins were transferred onto a polyvinylidene fluoride membrane (Millipore, Hertfordshire, UK) before blotting with human SOD1 (Calbiochem, Nottinghamshire, UK) or mouse tubulin antibody (Fisher, Leicestershire, UK).