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http://researchonline.ljmu.ac.uk/8734/

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Short title: Oxidative stress in SCA2

Full title: Evidence of oxidative stress and mitochondrial dysfunction in spinocerebellar ataxia type 2 (SCA2) patient fibroblasts: Effect of Coenzyme Q10 supplementation on these parameters

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Spinocerebellar ataxia type 2 (SCA2) is a rare neurodegenerative disorder caused by a CAG repeat expansion in the ataxin-2 gene. We show increased oxidative stress, abnormalities in the antioxidant system, changes in complexes involved in oxidative phosphorylation and changes in mitochondrial morphology in SCA2 patient fibroblasts compared to controls, and we show that treatment with CoQ10 can partially reverse these changes. Together, our results suggest that oxidative stress and mitochondrial dysfunction may be contributory factors to the pathophysiology of SCA2 and that therapeutic strategies involving manipulation of the antioxidant system could prove to be of clinical benefit.

Keywords: spinocerebellar ataxia type 2, SCA2, neurodegeneration, oxidative stress, mitochondrial function
1. Introduction

Spinocerebellar ataxia type 2 (SCA2) is a rare neurodegenerative disorder characterized clinically by cerebellar ataxia, weakness in the lower limbs, decreased muscle tone and vibration sense, postural tremor, dystonia, nystagmus, slow saccades and cognitive decline (Bird, 2015; Stefan M Pulst, 2015). It is the second-most prevalent spinocerebellar ataxia subtype worldwide, surpassed only by SCA3 (Durr, 2010) and it reaches the highest world prevalence rate in the Holguin province (Cuba) resulting from a putative founder effect (Velázquez Pérez et al., 2009). SCA2 is clinically heterogeneous with varying age of onset and disease severity, but in general as symptoms progress patients become increasingly dependent on others and have decreased life expectancy (Stefan M Pulst, 2015). SCA2 is genetically characterized by autosomal dominant inheritance of an expanded and meiotically unstable sequence of trinucleotide repeats (CAG) in exon 1 of the ataxin-2 gene (ATXN2) on chromosome 12q24.12 (Imbert et al., 1996; S M Pulst et al., 1996; Vinther-Jensen et al., 2012). ATXN2 comprises 14 to 31 CAG repeats in healthy individuals, while disease causing alleles comprise more than 33 CAG repeats (Stefan M Pulst, 2015). These CAG repeat expansions result in elongated polyglutamine domains in the gene product; ataxin-2 (S M Pulst et al., 1996). In the advanced clinical stages, the brains of SCA2 patients are neuropathologically characterized by intracellular protein aggregates and widespread neuronal loss in the thalamus, brainstem and cerebellum (Huynh, Figueroa, Hoang, & Pulst, 2000). Cerebellar Purkinje cells and several pontine, mesencephalic, and thalamic neurons are particularly affected by progressive neurodegeneration (Auburger, 2012).

Many aspects of the disease including epidemiology, pathology, genetics, and neuroimaging have been studied extensively. However, the precise mechanisms of the disease pathology are yet to be characterized. Various possible biochemical components of the SCA2 disease mechanism have been suggested. For example, it has been shown that mutant ataxin-
2 alters the activity of calcium and potassium channels in Purkinje cells, leading to apoptosis (A. Kasumu & Bezprozvanny, 2012; A. W. Kasumu et al., 2012). Other studies have shown involvement of the translational machinery, processing of RNA, and the endocytic machinery (Kozlov et al., 2001; M Ralser et al., 2005; Markus Ralser et al., 2005; Satterfield & Pallanck, 2006). Recently, mitochondrial dysfunction and oxidative stress have been shown to be involved in the pathology of several neurodegenerative diseases including Alzheimer disease (AD), Parkinson disease (PD), amyotrophic lateral sclerosis (ALS), Friedreich ataxia (FA) and Huntington disease (HD) (Federico et al., 2012), and although the mechanisms responsible for development of these diseases are not fully resolved, it seems clear that oxidative stress plays an important role. Furthermore, altered activity of key antioxidant enzymes and markers of increased apoptosis have been found in serum of SCA2 patients (Almaguer-Gotay et al., 2014; Swarup, Srivastava, Padma, & Moganty, 2013). However, there are few studies on oxidative stress in SCA2 and more are needed to understand the underlying mechanisms.

Mitochondria play many important roles in the cell, such as cell signalling, proliferation, differentiation, and cell death through apoptosis or necrosis (McBride, Neuspiel, & Wasiak, 2006). They are one of the major intracellular sources of reactive oxygen species (ROS), mainly originating from the protein complexes involved in oxidative phosphorylation (OXPHOS) (Dröse & Brandt, 2012; St-Pierre, Buckingham, Roebuck, & Brand, 2002). ROS produced continuously during normal cellular metabolism have essential functions in cellular signalling by regulating the expression/activity of many genes and enzymes, but the positive effect of ROS is highly dependent on the antioxidant system. If the level of ROS production overwhelms the antioxidant system, ROS becomes toxic to the cells resulting in oxidative stress and activation of mitochondrial quality control mechanisms to maintain mitochondrial function and cell viability (Jezek & Hlavatá, 2005; Valko et al., 2007). However, the exact
molecular causes of increased ROS and how mitochondrial mechanisms are initiated and compensate for physiological and pathological processes remain poorly understood.

In this study, we aimed to establish whether ROS production and overall mitochondrial dysfunction contribute to the pathophysiology of SCA2 by investigating cultured fibroblasts from SCA2 patients in comparison to age-matched controls. We show an increase in mitochondrial oxidative stress in patient fibroblasts as well as changes in mitochondrial morphology and respiratory chain (MRC) enzyme activity. We also show changes in the levels of key mediators of oxidative stress and mitochondrial function, both at the transcript and protein level. Furthermore, treatment with the antioxidant coenzyme Q10 (CoQ10) alleviated the oxidative stress and associated phenotypes in patient fibroblasts, suggesting that ROS and consequent mitochondrial dysfunction may be a key component in the pathogenesis of SCA2.
2. Experimental Procedures

2.1. Subjects and samples

Patients were recruited through the Neurogenetics Clinic, Danish Dementia Research Centre, Rigshospitalet, and written informed consent was obtained from all participating patients and control individuals. Skin biopsies were obtained from the forearm of five patients and three age matched controls of Danish origin and representative of the general Danish population. Biopsies were cut into small pieces and left untouched for 10 days in DMEM 1885 supplemented with 10% foetal bovine serum, 1% Glutamax (Gibco, Life Technologies) and 1% penicillin/streptomycin (BioSera, In Vitro) at 37°C, 5% CO$_2$ and 95% relative humidity. Subsequently, media was changed every 2-3 days and fibroblasts were expanded for further experiments.

2.2. Clinical assessment

A complete neurological examination was conducted and the clinical diagnosis of SCA2 was based on positive family history and disease manifestation such as ataxic gait, dysarthria, dysmetria, dysdiadochokinesia and slow saccades. The age at onset was defined as the onset of motor impairment as stated by the patient, and disease severity was assessed according to the Scale for Assessment and Rating of Ataxia (SARA) (Schmitz-Hubsch et al., 2006).

2.3. CAG repeat size determination

Genomic DNA was isolated from peripheral blood leukocytes or cultured fibroblasts and the $ATXN2$ CAG repeat length was assessed as previously described (Nielsen, Svenstrup, et al., 2012).

2.4. Cell culture
The fibroblasts were cultured in DMEM 1885 medium supplemented with 1% Glutamax, 10% FBS (In Vitro) and 1% penicillin/streptomycin at 37°C, 5% CO₂ 95% relative humidity. Cells treated with CoQ10 were incubated in standard medium containing 10 µmol/l CoQ10 (Sigma–Aldrich) for 7 days before harvest/measurements.

2.5. Mitochondrial ROS level measurements by MitoSOX

Mitochondrial superoxide levels were measured using the MitoSOX™ Red Mitochondrial Superoxide Indicator (MitoSOX) (Life Technologies) according to the manufacturer’s instructions. Briefly, cells were washed two times in PBS, and incubated at 37°C for 20 min in Hank’s balanced salt solution (HBSS) (Sigma-Aldrich) containing MitoSOX at 5 µM. Subsequently, cells were washed twice with PBS, trypsinized and re-suspended in Hoechst 33342 at 2 µg/ml and incubated for 15 min at 37°C for nuclear staining. Measurements of cellular fluorescence were performed on a NucleoCounter NC-3000 image cytometer (Chemometec), and subsequent analyses of cellular stress were performed using NucleoView 2.1 software (Chemometec). The Hoechst staining was used as masking channel to define cells to include into the analyses, and the cells showing oxidative stress were defined as cells displaying above-threshold superoxide levels compared to healthy control cell populations. Experiments were conducted in biological triplicates, and results were replicated in separate experiments for confirmation.

2.6. Intracellular hydrogen peroxide measurements

Intracellular hydrogen peroxide levels were measured using the intracellular hydrogen peroxide assay kit (Sigma, MAK164) according to the manufacturer’s instructions. Briefly, cells were washed two times in PBS, and incubated at 37°C for 30 min in assay buffer (HBSS) (Sigma-Aldrich) containing fluorescent hydrogen peroxide sensor (according to manufacturers’
instructions) and Hoechst 33342 at 2 µg/ml for nuclear staining. Measurements of cellular fluorescence were performed on a NucleoCounter NC-3000 image cytometer (Chemometec), and subsequent analyses of cellular stress were performed using NucleoView 2.1 software (Chemometec). The Hoechst staining was used as masking channel to define cells to include into the analyses, and the hydrogen peroxide level was defined by number of cells displaying above-threshold hydrogen peroxide levels compared to healthy control cell populations. Experiments were conducted in biological triplicates, and results were replicated in separate experiments for confirmation.

2.7. Visualization and quantification of mitochondrial morphology

Control and patient fibroblasts were incubated 30 min at 37°C with 100 nM MitoTracker Red CMXRos probe (Molecular Probes) in 10 cm² slide flasks (Nunc). Fibroblasts were fixed in 3.7% (w/v) paraformaldehyd (Sigma-Aldrich) for 10 min, washed in PBS before cover slipping with Prolong Gold antifade mounting media containing DAPI (Life Technologies). Fluorescence microscopy was conducted on an Olympus IX73 microscope using the 40x objective. Quantification of cell morphologies was conducted using the MiToBo plugin for FIJI. Briefly, the MultiChannelParticleAnalyzer2D was used to generate thresholded pictures of the particles, and the number of large elongated mitochondrial structures (size above 50 pixel^2 and a circularity of 0.00-0.50) per cell was then counted using “Analyze Particles” in FIJI in order to obtain a measure of the degree to which the cells contain threadlike mitochondrial organization. Cells were then divided into three groups (normal, intermediate and punctate) according to the number of elongated mitochondrial structures they contained. All quantification was conducted by a researcher blind to cell type (control vs. SCA2). All results were replicated in separate experiments to verify findings.
2.8. Quantitative PCR

Total RNA was isolated from fibroblasts using NucleoSpin RNA isolation kit (Macherey-Nagel) and on-column DNase treatment to remove any remaining DNA contamination.

Complementary DNA (cDNA) was synthesized from 0.5 μg RNA using Superscript II cDNA synthesis kit (Life Technologies) with random hexamer primers. ApoE (Hs00171168_m1), CAT (Hs00156308_m1), mTOR (Hs00234508_m1), SIRT3 (Hs00953477_m1), PRKAA1 (Hs01562315_m1), SOD2 (Hs00167309_m1), HIF1α (Hs00153153_m1), MFN1 (Hs00966851_m1), DRP1 (Hs01552605_m1) and TFAM (Hs01082775_m1) mRNA amounts were measured relative to Ribonuclease P protein subunit p29 (POP4) (Hs00198357_m1) (Life Technologies) and measured in triplicates by quantitative real-time PCR using the CFX96 Real Time System (BioRad) and TaqMan probe chemistry (Applied Biosystems). All probes used were conjugated to the 6-FAM dye at the 5’ end and a non-fluorescent quencher group in the 3’ end. Results were calculated using the relative standard curves method and normalized to the non-CoQ10-treated control cells. Experiments were conducted in biological triplicates, and results were replicated in separate experiments to confirm findings.

2.9. Western Blots

Protein levels in lysates of control and SCA2 patient fibroblasts were determined via western blot. Cells treated for seven days either with DMSO vehicle or 10 μM CoQ10 dissolved in DMSO were washed twice with PBS and collected by scraping on day of lysis. Cells were lysed using CelLytic M Buffer (Sigma-Aldrich) containing cOmplete protease and phosphatase inhibitors (Roche) and protein content of lysates was measured via Bradford assay (ref??). Gel electrophoresis of 15 μg of protein per lane was performed using 12 well 10% polyacrylamide Bis-tris gels (Life Technologies) in a Xcell SureLock electrophoresis system (ThermoFisher) at 150v for 90 min. Transfer to nitrocellulose membranes was carried out in transfer buffer (Life
technologies) containing 20% ethanol for 90 min in the same Xcell system. Membranes were blocked for 60 minutes at room temperature in blocking buffer (5% Nonfat dry milk or 3% BSA in TBST) followed by overnight incubation with primary antibodies (α-tubulin, apolipoprotein E, catalase, dynamin-related protein 1, glyceraldehyde-phosphate dehydrogenase (GAPDH), mitofusin 1, mitochondrial transcription factor 1, superoxide dismutase 2 (Abcam)) in blocking buffer at 4°C. Blots were washed in TBST and incubated with horseradish-peroxidase-coupled secondary antibodies (Dako) in blocking buffer for 60 min at room temperature. Blots were visualized using ECL select reagent (GE Healthcare) on a GENESys chemiluminescent imaging system (Syngene) and densiometric quantification of bands was carried out using Genetools software (Syngene). All quantification of protein levels was normalized to concurrent loading control bands (either GAPDH or α-tubulin depending on molecular weight of target protein) after which individual patient fibroblast protein levels were normalized against concurrent average control fibroblast protein levels.

2.10. Determination of CoQ10 levels in cultured skin fibroblasts

Total cellular CoQ10 status was determined in fibroblasts by reversed-phase HPLC coupled to UV detection at 275 nm as described previously by Duncan et al (Duncan et al., 2005). The CoQ10 levels were expressed as pmol/mg of protein.

2.11. Assessment of mitochondrial respiratory chain enzyme activities

MRC Complex I (NADH:ubiquinone reductase; EC 1.6.5.3); Complex II/III (succinate: cytochrome c reductase; EC 1.3.5.1 + EC 1.10.2.2) and IV (cytochrome c oxidase; EC 1.9.3. 1) activities were determined spectrophotometrically according to the methods described by
Duberley et al. (2013). Citrate synthase activity was determined according to the method described by Duberley et al. (2013).

All MRC activities were expressed as ratios to citrate synthase EC 2.3.3.1; mitochondrial marker enzyme) activity to account for differences in the mitochondrial content as previously described (Selak et al., 2000).

2.12. Statistical analyses

Student’s t-test was used to determine significant differences when comparing the group of healthy controls to the group of SCA2 patient cells. One-column t-test was used to test the difference in oxidative stress upon CoQ10 treatment. One-way ANOVA tests with Dunnet’s Multiple Comparison Post-hoc tests were performed to determine significant differences between control cells and SCA2 patient cells when looking at individual cell lines. Differences in morphological distribution between the two groups were tested using a χ²-test. Differences were considered significant when p-values below 0.05 were obtained.
3. Results

3.1. Patients

We obtained fibroblast cultures from three healthy controls and five SCA2 gene expansion carriers, one of whom was pre-symptomatic at the time of biopsy. All five individuals were subjected to thorough clinical evaluation. The four symptomatic patients presented with classic signs of SCA2 including broad-based gait, slow saccades and dysarthria. Molecular genetic testing of all five individuals showed that the length of the CAG repeat in the elongated allele varied between 36 and 44 CAG repeats. The clinical information is summarized in Table 1.

3.2. Fibroblasts express ataxin-2

Choosing fibroblasts derived from forearm skin biopsies as our model system to study oxidative stress in SCA2, we first determined whether this cell type expresses ataxin-2. Using western blotting, we found that all fibroblast lines abundantly expressed ataxin-2 at comparable levels (results not shown). The repeat lengths of ATXN2 were determined for all cultured fibroblast lines to confirm that there were no changes in CAG-repeat lengths due to cell culturing.

3.3 Oxidative stress

3.3.1 Mitochondrial oxidative stress and antioxidant response in patient fibroblasts

SCA2 has been suggested to be linked to oxidative stress, yet the source of oxidative stress in patients is not well characterized (Almaguer-Gotay et al., 2014; Swarup et al., 2013). In this study we investigated the level of mitochondrial oxidative stress by assessing superoxide levels in fibroblasts from SCA2 patients and controls. Cells grown in parallel were assessed for mitochondrial superoxide production by measuring MitoSOX fluorescence intensity. SCA2 cells
displayed significantly increased amount of oxidative stress compared to control cells (28.1±2.5% vs 1.5±0.5% stressed cells for SCA2 and control lines, respectively, p<0.0001) (Figure 1A).

Since previous studies have shown that moderately decreased levels of CoQ10 can be associated with increased levels of oxidative stress (Quinzii et al., 2010), we examined the level of CoQ10 in the fibroblasts. However, we did not find any significant decrease in the CoQ10 levels in untreated SCA2 fibroblasts relative to untreated control fibroblasts (p=0.21) (Figure 1B).

In addition to its traditional role as an electron transporter in the electron transport chain, CoQ10 in its reduced form, ubiquinol, is recognized as an antioxidant and free radical scavenger, protecting membrane lipids, proteins, and mitochondrial DNA against oxidative damage (Mancuso, Orsucci, Volpi, Calsolaro, & Siciliano, 2010). We therefore tested whether treatment with CoQ10 alleviated the increased oxidative stress found in the patient fibroblasts. As expected, we found that the CoQ10 levels increased dramatically in the cells upon CoQ10 treatment (Figure 1B). More importantly, we found that treatment with CoQ10 significantly reduced the number of cells showing oxidative stress by 31.4±7.0% (p<0.001) in patients, whereas control fibroblasts were unaffected by the treatment (p=0.82) (Figure 1C).

In response to oxidative stress, cells regulate their antioxidant system in order to avoid damage to proteins, lipids and DNA (Circu & Aw, 2010). The enzyme manganese superoxide dismutase (mnSOD, encoded by the gene SOD2) is an essential mitochondrial antioxidant, which makes up the first line of defense against oxidative stress by converting superoxide into hydrogen peroxide (H₂O₂) (Miao & St Clair, 2009). Subsequently, the enzyme catalase (CAT), among others, decomposes the cellular H₂O₂ into water and oxygen thereby eliminating the effect of this highly damaging reactive species (Circu & Aw, 2010). To investigate whether patient fibroblasts regulate the antioxidant system in response to cellular
stress, we measured SOD2 and CAT mRNA expression by quantitative RT-PCR. We found over twofold increase in the SOD2 mRNA levels of the SCA2 fibroblasts relative to controls (p=0.0002) (Figure 2A), consistent with a cellular response to the increased oxidative stress revealed by MitoSOX analyses. Subsequent western blot analysis showed changes in protein levels of superoxide dismutase consistent with observed changes in mRNA, with significantly increased superoxide dismutase protein in SCA2 fibroblasts (approximately 130% increase p=0.013) (Figure 2C,E). Conversely, CAT mRNA levels were significantly decreased in SCA2 fibroblasts compared to controls (a decrease of approximately 20%, p=0.0026) (Figure 2B). These results were also replicated at the protein level, with an approximate decrease of 41% in catalase protein levels in SCA2 vs. control fibroblasts (p=0.0004) (Figure 2D,F).

To examine whether regulation of mRNA levels of these enzymes is connected to increased oxidative stress, we also measured mRNA levels of SOD2 and CAT in patient and control fibroblasts after CoQ10 treatment. The results showed that the CoQ10 treatment reversed changes in SOD2 mRNA levels, but did not reverse changes in CAT mRNA levels, which continued to show a decrease of approximately 20% in patient cells (p=0.0011) (Figure 2A,B)). These results were once again replicated at the protein level for both superoxide dismutase, which showed reversal of differences in protein levels when treated with CoQ10, and for catalase, which continued to show a decrease in levels in patient cells (approximately 30%, p=0.0045) (Figure 2C-F).

With increased levels of superoxide dismutase likely producing increased levels of hydrogen peroxide, and decreased levels of catalase likely unable to eliminate this increased peroxide burden, increased levels of intracellular hydrogen peroxide would be expected. To examine this, we measured intracellular hydrogen peroxide levels in control and patient fibroblasts, in the absence and presence of CoQ10. Untreated patient cells showed a significant increase in cells containing increased hydrogen peroxide levels (approximately 61%,
p<0.0001), while treatment with CoQ10 eliminated this difference in hydrogen peroxide content between control and patient cells (Figure 1D).

3.3. Mitochondrial function

3.3.1. Regulation of mitochondrial dynamics

Although the details of mitochondrial dynamics are still unclear, it is known that mitochondrial fusion and fission events are crucial for mitochondrial maintenance (Westermann, 2010). Fusion and fission enable the cells to remove damaged mitochondria, and it has been shown that cells regulate these events during oxidative stress by inhibiting the fusion of mitochondria to prevent damaged and healthy mitochondria from fusing. Instead, damaged mitochondria are targeted for degradation by means of mitochondria-specific autophagy known as mitophagy (Ashrafi & Schwarz, 2013; Liu, Weaver, Shirihai, & Hajnóczky, 2009; Twig et al., 2008). Since our data show significant increase in the level of oxidative stress in the SCA2 patient fibroblasts, we evaluated mitochondrial morphology and dynamics in these cells.

In healthy cells mitochondria typically surround the nucleus in a threadlike pattern, whereas punctate mitochondrial structure is indicative of mitochondrial damage and cellular stress (Bereiter-Hahn, 2014). To investigate morphological changes, we labeled the mitochondria using MitoTracker Red CMXRos and analyzed the fibroblasts by fluorescence microscopy. We found that control cells mostly displayed mitochondria with threadlike (denoted “normal structure”) structure (Figure 3A), whereas SCA2 cells displayed either a mixture of punctate and threadlike mitochondria (denoted intermediate structure) (Figure 3B) or punctate mitochondria only (Figure 3C) (see Experimental procedures for details). In 69.5±12.0% of the control cells mitochondria displayed normal thread-like morphology with little intermediate or punctate morphology. In contrast, 41.9±5.3% and 31.8% of patient fibroblasts displayed intermediate and punctate structure, respectively (Figure 3D). Hence, the
overall distribution of the mitochondrial morphological phenotypes was significantly different between control and SCA2 fibroblasts (p<0.0001), supporting the hypothesis that oxidative stress influences mitochondrial dynamics in SCA2.

To elucidate whether altered mitochondrial morphology in SCA2 patient fibroblasts results in altered mitochondrial amount, we analyzed the cellular mitochondrial content by assessment of the activity of the mitochondrial marker enzyme citrate synthase (CS) (Hughes et al., 2014). There was no change in CS activity between patient and control fibroblasts (Figure 3E), indicating that the mitochondrial content was not significantly different in patient and control fibroblasts (p=0.25). In concurrence with this measure of mitochondrial content, we measured the mRNA levels of TFAM (transcription factor A, mitochondrial). TFAM is a DNA-binding protein that activates transcription of mitochondrial DNA and it is involved in mitochondrial biogenesis, packaging and organization of the mitochondrial genome (Bonawitz, Clayton, & Shadel, 2006; Ngo, Kaiser, & Chan, 2011). By investigating the TFAM mRNA levels it is possible to evaluate whether there is transcriptional regulation of the mitochondrial content in SCA2 fibroblasts compared to controls. We found a significant upregulation of TFAM mRNA levels (10%, p=0.007) in the patient fibroblasts compared to the controls, indicating that there is transcriptional upregulation of mitochondrial content in these cells (Figure 4A). By treating SCA2 fibroblasts with CoQ10, TFAM mRNA levels were normalized (p=0.06), although the tendency to increased TFAM mRNA expression remained (Figure 4A), further supporting the hypothesis that TFAM mRNA levels are regulated in response to oxidative stress. These trends were replicated at the protein level, with apparent increases in TFAM protein in SCA2 cells both in the absence and presence of CoQ10, though neither of these results was statistically significant (Figure 4D, G). As mitochondrial content as measured by CS activity does not vary significantly between patient and control fibroblasts, mitochondrial turnover rate may be involved in maintaining this steady number of mitochondria. To investigate this, we also
assessed the mRNA levels and protein levels of mitofusin 1 (MFN1) and dynamin-related protein 1 (DRP1), which are involved in mitochondrial fusion and fission, respectively (Legros, Lombès, Frachon, & Rojo, 2002). mRNA levels of both MFN1 and DRP1 showed significant increases (both approximately 20%, p=0.013, P=0.006 respectively) in SCA2 fibroblasts versus controls (Figure 4B,C). With CoQ10 treatment the mRNA levels of these transcripts in patient fibroblasts were no longer significantly higher than control levels (Figure 4B,C). Finally, results from immunoblot assessment of protein levels showed trends similar to the changes seen at the mRNA level for both mitofusin1 and dynamin related protein 1 (approximately 60% and 20% increase in SCA2 cells, respectively), though these changes were not significant (p=0.07 and p=0.30 respectively) (Figure 4E,F,H,I).

3.4.2. Mitochondrial electron transport chain enzyme activity

To assess the effect of oxidative stress on mitochondrial metabolism, we measured the enzymatic activity of the mitochondrial electron transport chain complexes I, II-III and IV. We found that enzymatic activity of complex I was significantly reduced approximately 32% in SCA2 cells relative to controls (p=0.014). We also found that the enzymatic activity of complex II-III was significantly reduced by approximately 50% in SCA2 fibroblasts compared to controls (p=0.005), whilst complex IV activity showed no significant difference (p=0.34) (Figure 5A-C). Upon treatment with CoQ10, complex I and II-III activity of the SCA2 fibroblasts was no longer significantly different than that of controls (Figure 5A-B), suggesting a functional association of mitochondrial metabolism with oxidative stress-related cellular processes.

3.4. Transcriptional regulation of proteins involved in oxidative stress and neurodegeneration

In addition to the direct response by cells upon oxidative stress, e.g. levels of enzymes involved in scavenging and protecting against reactive oxygen species, other transcriptional changes
may occur as downstream effects of a stressed cellular state. We therefore analyzed mRNA levels of some major transcriptional regulators including mTOR (mammalian target of rapamycin), HIF1α (hypoxia-inducible factor 1-alpha), SIRT3 (mitochondrial NAD-dependent deacetylase sirtuin-3) and PRKAA1 (5′-AMP-activated protein kinase catalytic subunit alpha-1) (Canal, Román-Aumedes, Martín-Flores, Pérez-Fernández, & Malagelada, 2014; Krishan, Richardson, & Sahni, 2014; Olsen, Cornelius, & Gregersen, 2015). These four genes have previously been shown to be involved in various neurodegenerative diseases as well as transcriptional regulation/metabolic shifting (Warburg effect) as a result of oxidative stress (Olsen et al., 2015).

Our analysis showed that the mRNA level of mTOR is significantly upregulated (18%, p=0.027) in SCA2 fibroblasts relative to controls (Figure 6A). HIF1α, SIRT3 and PRKAA1 were not significantly changed in patient cells (p=0.062, p=0.67 and p=0.75, respectively) (Figure 6B,D,E). Treatment with CoQ10 reversed mTOR mRNA levels to normal in SCA2 cells, but did not have any significant effect on HIF1α SIRT3 or PRKAA1 mRNA levels (Figure 6B,D,E). In the case of mTOR, phosphorylation-state-dependent activity plays a very important role in its function, and its’ downstream targets are too diverse. In order to draw specific conclusions on mTOR function in this system, more detailed studies would be required than assessment of overall protein levels or phosphorylation status. In the case of HIF1α, SIRT3 and PRKAA1, lack of significant changes at the mRNA level precluded the value of further investigation at the protein level (see discussion).

A recent proteomic study on SCA2 patient blood plasma showed a large increase in the apolipoprotein E (ApoE) protein level (Swarup et al., 2013). In addition to being involved in lipid and cholesterol transport and metabolism, ApoE has previously been shown to be protective against oxidative damage by ROS such as H2O2, which we see increased in our SCA2 patient cells (Miyata & Smith, 1996; Tangirala et al., 2001). Based on these results, we
investigated the ApoE mRNA levels in SCA2 patient fibroblasts. We found that ApoE expression was increased approximately 2.5-fold (p<0.0001) in patient fibroblasts compared to controls (Figure 6C). Finally, CoQ10 treatment reversed ApoE mRNA levels to control levels (Figure 6C), suggesting that ApoE is regulated in response to oxidative stress in patient fibroblasts. These changes in mRNA levels were replicated at the protein level, where SCA2 patient fibroblasts showed an increase of approximately 3.5 fold vs. control fibroblasts (p=0.0224), changes that were reversed in the presence of CoQ10 (Figure 6F,G).

4. Discussion

In this study, we investigated five individuals with expanded CAG repeats in ATXN2 and demonstrated involvement of oxidative stress and general mitochondrial dysfunction in cultured fibroblasts. Patient-derived fibroblasts have previously been used to examine general cellular pathways, such as mitochondrial, lysosomal, and autophagic dysfunction in neurodegenerative diseases such as Parkinson disease, Huntington disease and frontotemporal dementia (Erie, Sacino, Houle, Lu, & Wei, 2015; Lippolis et al., 2015; Nielsen, Mizielinska, Hasholt, Isaacs, & Nielsen, 2012; Urwin et al., 2010). In some cases the key pathogenic mechanisms of neurodegeneration have been faithfully replicated in fibroblasts (Clayton et al., 2015; Urwin et al., 2010), so although a non-neuronal cell type, patient-derived fibroblasts have been shown to be relevant cellular models to study pathogenic mechanisms of neurodegeneration. In the present study, we investigated the general cellular pathway of oxidative stress in SCA2, and our findings suggest the presence of a complex system wherein the cells regulate their antioxidant system, mitochondrial dynamics and energy metabolism, leading to changes in transcriptional regulation most likely to minimise the consequences of the oxidative stress.
4.1. Oxidative stress and mitochondrial antioxidant response in SCA2 patient fibroblasts

In this study we showed a significant increase in mitochondrial superoxide production in patient fibroblasts, which indicates that the cells of these patients have an increased basal level of chronic oxidative stress that might contribute to the cellular pathogenesis of SCA2. This increased level of superoxide leads to increased intracellular levels of hydrogen peroxide in SCA2 patient cells, and the levels of both of these oxidative species were at least partially alleviated with CoQ10 treatment. Considering that there was no significant decrease in the basal level of CoQ10 in control versus patient fibroblasts, it appears that increased levels of reactive oxygen species rather than a primary CoQ10 deficiency, are responsible for the downstream oxidative stress effects observed in the proceeding experiments. Therefore, it appears that CoQ10 functions as a primary oxidative stress scavenger.

We examined cellular antioxidant gene expression in response to oxidative stress in order to understand such defense mechanisms in SCA2. We found increased superoxide dismutase expression at both the transcript and protein level consistent with increased need for scavenging superoxide. These increases were reversed in patient fibroblasts following CoQ10 treatment, indicating that expression of this enzyme was indeed regulated as a response to oxidative stress. However, catalase mRNA and protein expression was decreased in patient fibroblasts and remained unchanged with CoQ10 treatment. This lack of response indicates that changes in catalase levels may not be directly linked to oxidative stress. As catalase enzyme levels would be expected to increase with increased cellular levels of ROS, it is likely that the decrease in catalase levels that we observe is related to other processes in these cells. For example, decreased catalase expression is frequently associated with disease states, e.g. in patients with dementia (Gsell et al., 1995) and in different forms of cancer (Glorieux et al., 2014). Our data suggested that H$_2$O$_2$ may not be managed properly in the cells, leading to increased H$_2$O$_2$ concentration acting directly as part of the pathogenic mechanism. This was
confirmed by intracellular hydrogen peroxide assay, where we found that a greater number of SCA2 patient cells showed increased \( \text{H}_2\text{O}_2 \) levels relative to controls, and these changes were alleviated by treatment with CoQ10. These data suggest that there are multiple sources of damaging oxidative species present in the SCA2 fibroblasts, all of which respond to antioxidant treatment, and which may have important pathogenic roles in the SCA2 disease phenotype.

4.2. Regulation of mitochondrial dynamics

Mitochondrial morphology was significantly different in patient fibroblasts compared to controls. Control cells showed more threadlike mitochondrial morphology, while in patient cells, mitochondrial structures were punctate. The increased number of cells with punctate mitochondrial structures in the patient fibroblasts suggests a change in the dynamic processes of mitochondrial fusion and fission. It has been shown that the cell protects the mitochondria in the presence of high levels of oxidative stress by inhibiting mitochondrial fusion, therein preventing fusion between healthy and damaged mitochondria. In these cases, damaged mitochondria are targeted instead for degradation by means of mitophagy (Ashrafi & Schwarz, 2013; Liu et al., 2009; Twig et al., 2008). Such increased mitophagy would most likely be accompanied by increased biogenesis of mitochondria to maintain the number of mitochondria in the cell. Citrate synthase levels indicated similar mitochondrial content in the patient and control cells, despite differences in morphology. To determine whether there is a compensatory increase in mitochondrial biogenesis in the SCA2 patient cells to replace mitochondria lost to mitophagy, we measured the expression of \( \text{TFAM} \) mRNA and corresponding protein in the cells. TFAM is a DNA-binding protein that activates transcription of mitochondrial DNA; it is also responsible for coating and packaging the mitochondrial genome (Bonawitz et al., 2006; Ngo et al., 2011), and changes in TFAM levels therefore indicate transcriptional regulation of the mitochondrial number. The slight increase in \( \text{TFAM} \)
mRNA, that we observe could suggest increased mitochondrial biogenesis, and in the context of unchanged mitochondrial content as indicated by CS activity, could imply that the cells are eliminating mitochondria via mitophagy at the same rate as they are being formed. The TFAM protein levels showed the same trend towards increased expression, although the changes did not meet statistical significance. To better understand these dynamic processes, we examined the levels of MFN1 and DRP1 mRNA and protein, which are involved in mitochondrial fusion and fission, respectively. We found that mRNA for both of these proteins was increased significantly, and protein levels trended in this direction as well, although they were not significant. Though these changes in mitochondrial dynamics are small, combined with the data on mitochondrial morphology, citrate synthase and gene expression suggest that this aspect of mitochondrial maintenance could be a process worthy of future studies of the SCA2 pathological mechanism. Furthermore, previous studies investigating oxidative stress and mitophagy have revealed increased fragmentation of mitochondria via mitochondrial fission in response to ROS, and that the resultant mitophagy was DRP-1 dependent. The increase in punctate mitochondrial morphology, increased ROS levels, and increased DRP1 expression we observe in the SCA2 patient fibroblasts support this model of oxidative stress-mediated mitochondrial maintenance.

4.3. Mitochondrial metabolism and the electron transport chain

To better understand the involvement of the components of the electron transport chain and the relationship between mitochondrial metabolism and oxidative stress, we measured the enzymatic activity of complexes I, II+III and IV. While complex IV activity was unchanged, there was a significant decrease in activity of complexes I and II-III in SCA2 patient fibroblasts relative to controls. The decrease in complex I and II-III activity could be a protective mechanism decreasing mitochondrial metabolism to prevent further superoxide production (part of the
The Warburg effect discussed later), or it could be attributed to the oxidative stress itself, as superoxide and other ROS are known to impair cellular metabolism (Beal, 1996). Finally, the fact that these effects were either partially (complex II+III) or completely (complex I) alleviated by CoQ10 supplementation indicates that the observed metabolic changes may be secondary to oxidative stress.

4.4. Transcriptional regulation of proteins involved in oxidative stress and neurodegeneration

mTOR is an established transcriptional regulator, known to control apoptosis and autophagy in the cells of the nervous system (Ghavami et al., 2014). Precise regulation of mTOR has proven extremely important in neurons, where even small changes can lead to neurodegeneration (Canal et al., 2014). In our study, SCA2 patient fibroblasts show a modest but significant increase in mTOR mRNA levels, which seem to be regulated in response to oxidative stress, as indicated by the normalization of the mTOR mRNA level after CoQ10 treatment. Due to the very complex regulation of mTOR and its downstream targets, we did, however, not attempt to assess these changes at on the protein level, since without investigating such complex regulation (e.g. post-translational modifications), which falls outside the scope of this study, we cannot draw conclusive links between mTOR and SCA2. However, in AD hyperactive mTOR has been correlated with disease pathology, leading to impairment of autophagy and thereby Aβ and Tau accumulation (Cai, Chen, He, Xiao, & Yan, 2015; Wang et al., 2014). Therefore, a link between mTOR regulation and SCA2 thus warrants further study.

Cells affected by oxidative stress respond via a number of different mechanisms to minimize damage caused by ROS. One of these mechanisms is known as the Warburg effect, which is a metabolic shift from oxidative phosphorylation to glycolysis that minimizes the activity of the electron transport chain (the main source for oxidative species in the cell), to ensure cellular maintenance and proliferation during oxidative stress. To investigate the
Warburg effect in our model, we measured the expression of PRKAA1, SIRT3, and HIF1α, which are known regulators of cellular metabolism governing the Warburg effect. However, we did not find any changes in the mRNA level in the patient fibroblasts as compared to the controls for any of the three transcripts. In conclusion, although mTOR and mitochondrial complex II-III activity may indicate some degree of metabolic shifting, other factors controlling metabolism do not (PRKAA1, SIRT3 and HIF1α), and thus such metabolic shifting is probably not occurring to a large extent in SCA2 fibroblasts.

A classic hallmark of SCA2 pathology is intracellular inclusions consisting of aggregated proteins, most notably expanded ataxin-2. In a range of other neurodegenerative diseases, protein aggregation is also a prominent pathological characteristic, for example in AD. In these cases, certain polymorphisms in ApoE have been found to be potent risk factors, although this mechanism is currently unclear (Corder et al., 1993). It has been hypothesized that ApoE is an amyloid catalyst or “pathological chaperone” (Bales et al., 1999; Ma, Yee, Brewer, Das, & Potter, 1994; Wisniewski, Castaño, Golabek, Vogel, & Frangione, 1994), and it has been posited that ApoE regulates Aβ clearance (Castellano et al., 2011; Cramer et al., 2012; Jiang et al., 2008). Notably, it has previously been shown that ApoE is increased in serum of SCA2 patients (Swarup et al., 2013). Consistent with this finding we found markedly increased levels of ApoE mRNA and protein in SCA2 patient fibroblasts compared to controls, and this difference was reversed upon treatment with CoQ10. These results suggest that the increase in ApoE may in fact be a response to oxidative stress. The present increased ApoE levels concurrent with increased levels of ROS in SCA2 patient lines is noteworthy, since ApoE has also been shown to protect against oxidative stress insults including H₂O₂ in in vitro and in vivo in models of atherosclerosis (Miyata & Smith, 1996; Tangirala et al., 2001). Furthermore, it has been suggested that ApoE protects against neurodegeneration caused by oxidative stress.
by binding to peroxidated lipids (Pedersen, Chan, & Mattson, 2000). All of these data support a role of ApoE in the protection against oxidative stress and neurodegeneration.

4.5. CoQ10 treatment

In our study, we have shown reversal of several of the cellular changes related to mitochondrial dysfunction by treating cells with the antioxidant CoQ10. In a recent study, it was shown that CoQ10 treatment was associated with better outcomes as indexed by the SARA score and Unified Huntington’s Disease Rating Scale (UHDRS-IV) score in a cross-sectional study in SCA1 and SCA3, whereas the outcome for SCA2 patients had a tendency of improvement that did not reach statistical significance (Lo et al., 2015). However, CoQ10 treatment did not influence the outcome in a 2-year longitudinal study in any of the disorders. These results are not fully conclusive as to whether or not CoQ10 treatment is beneficial in SCA2 or other SCAs, but together with our data on the cellular phenotypes in SCA2 it suggests further studies on mitochondrial involvement in the pathology of SCAs are needed to assess the possibility for applying treatment strategies that modulate the antioxidant system. Given the low bioavailability of CoQ10 (Bank, Kagan, & Madhavi, 2011) it might be necessary to use different formulations of CoQ10 or even use novel compounds with similar antioxidant properties but with better bioavailability in order to obtain efficacy for such treatment regimes.

4.6. Conclusion

In conclusion, we have shown evidence of mitochondrial-specific oxidative stress and mitochondrial dysfunction in SCA2 fibroblasts for the first time, which may be factors to consider in the pathophysiology of this disorder. Another important finding of this study is that one of the SCA2 fibroblast lines with an expanded CAG repeat showed evidence of increased
oxidative stress/mitochondrial dysfunction despite coming from a patient who has yet to exhibit any clinical symptoms. This indicates that the oxidative stress and mitochondrial dysfunction may precede disease onset. Finally, the alleviation of many of these oxidative stress-related phenotypes by CoQ10 treatment could indicate that the cellular antioxidant system and its regulation are potential therapeutic targets in this as-yet untreatable disease, but further investigations are required to identify possible therapeutic targets.
5. Acknowledgments

We would like to thank the patients and their families for participating in this study.

Furthermore, we want to thank Lis Hasholt (Department of Cellular and Molecular Medicine, University of Copenhagen, Denmark) for valuable help with establishing fibroblast cultures.
6. Ethics statement

The Ethics Committee of The Capital Region of Denmark approved the study (H-4-2011-157), and all participating individuals gave written informed consent before participating.
7. Funding

This work was supported by the Novo Nordisk Foundation; the Aase and Ejnar Danielsen Foundation and the Lundbeck Foundation, the National Institute for Health Research University College London Hospitals Biomedical Research Centre. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
8. Conflicts of interest

The authors declare to have no conflicts of interest.
9. References


http://doi.org/10.1016/j.molcel.2006.11.024

http://doi.org/10.2147/NDT.S75717

http://doi.org/10.3389/fncel.2014.00313

http://doi.org/10.1126/scitranslmed.3002156

Laboratory Investigation; a Journal of Technical Methods and Pathology, 90(5), 762–73.
http://doi.org/10.1038/labinvest.2010.36


Hughes, S. D., Kanabus, M., Anderson, G., Hargreaves, I. P., Rutherford, T., O’Donnell, M., ...


Wong, M. Mammalian target of rapamycin (mTOR) pathways in neurological diseases.

Figure Legends:

**Fig. 1**

**ROS generation in SCA2 patient fibroblasts.** (A) Oxidative stress levels were determined by image cytometry using the mitochondrial superoxide probe (MitoSOX) in fibroblast cultures from SCA2 patients and age matched controls. Results are expressed as percentages of unstressed (US) and stressed (S) cells as defined by MitoSOX intensity of above threshold level defined in healthy control cells. *** denotes statistical significance, p<0.001 (Student’s t-test).

(B) CoQ10 levels in cells from patients and controls with or without 7 days of treatment with 10 µM CoQ10. (C) Oxidative stress levels in with or without 7 days with 10 µM CoQ10 treatment. Results are expressed as the proportion of stressed cells after CoQ10 treatment in either control or patient cell lines, relative to vehicle treatment. This indicates that CoQ10 has no effect on stressed cells in control lines, while significantly decreasing proportion of stressed cells in patient cell lines. *** denotes statistical significance, p-value < 0.001 (Student’s t-test).

(D) Proportion of cells showing increased intracellular hydrogen peroxide levels relative to controls in untreated fibroblasts and in fibroblasts treated for 7 days with 10 µM CoQ10. *** denotes statistical significance, p<0.001

**Fig. 2**

**Antioxidant enzyme levels in SCA2 patient fibroblasts.** (A) and (B) mRNA levels of SOD2 and CAT as measured by quantitative PCR with and without CoQ10 treatment. CoQ10 treatment was conducted for 7 days with 10 µM CoQ10. *** and ** denotes statistical significance, p-values of < 0.001 and < 0.01, respectively (Student’s t-test).

(C) and (D) Protein levels of superoxide dismutase and catalase as measured by immunoblot with and without CoQ10
treatment. CoQ10 treatment was conducted for 7 days with 10 µM CoQ10. * and ** denotes statistical significance, p-values of < 0.05 and < 0.01, respectively (Student’s t-test). (D) and (E) representative immunoblots of superoxide dismutase and catalase with respective GAPDH loading controls represented.

**Fig. 3**

**Mitochondrial morphology.** (A-C) Patient fibroblasts stained with MitoTracker Red CMXRox (orange) and DAPI (blue) to visualize mitochondrial morphology: (A) Mitochondria showing normal “thread-like” structure (B) Mitochondria showing intermediate structure of thread-like and punctate structures. (C) Mitochondria showing punctate structure. (D) Quantification of mitochondrial morphology distribution in SCA2 patient and control fibroblasts using the above-defined morphological groupings: Normal (N), intermediate (I) and punctate (P). *** denotes statistical significance, p<0.001 (χ²-test). (E) Mitochondrial mass as analyzed by citrate synthase enzymatic activity (nmol/min/mL).

**Fig. 4**

**Mitochondrial regulation and dynamics.** mRNA levels of TFAM (A), MFN1 (B) and DRP1 (C) as measured by quantitative PCR. For CoQ10 treatments fibroblasts were treated for 7 days with 10 µM CoQ10. * and ** denotes statistical significance, p-values of < 0.05 and < 0.01, respectively (Student’s t-test). Protein levels of mitochondrial transcription factor A (D), mitofusin 1 (E) and dynamin related protein 1 (F) as measured by immunoblot. For CoQ10 treatments fibroblasts were treated for 7 days with 10 µM CoQ10. (G), (H), and (I) representative immunoblots of mitochondrial transcription factor A, mitofusin 1 and catalase, respectively, with respective α-tubulin loading controls represented.
Fig. 5.

**Analysis of mitochondrial metabolism.** (A) Enzymatic activity of mitochondrial electron transport complex I. * denotes statistical significance, \( p<0.05 \). (B) Enzymatic activity of mitochondrial electron transport complex II-III. ** denotes statistical significance, \( p<0.01 \). (C) Enzymatic activity of mitochondrial electron transport complex IV. Enzyme complex activities are expressed as a ratio to citrate synthase activity. CoQ10 treated cells were treated for 7 days with 10 µM CoQ10. Student’s t-test was used for statistical testing.

Fig. 6.

**Transcriptional regulation and apolipoprotein E in SCA2 patient fibroblasts.** mRNA levels of mTOR (A), HIF1a (B), ApoE (C), PRKAA1 (D) and SIRT3 (E) as measured by quantitative PCR. * and *** denotes statistical significance with p-values of \( p<0.05 \) and \( p<0.001 \), respectively. Protein levels of apolipoprotein E (E) as measured by immunoblot. * denotes statistical significance with p-values of \( p<0.05 \). CoQ10 treated cells were treated for 7 days with 10 µM CoQ10. Student’s t-test was used for statistical testing. (G) representative immunoblots of apolipoprotein E with respective α-tubulin loading controls represented.

Table 1.

**Clinical information on the participating patients**

<table>
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¹ Scale for assessment and rating of ataxia (24).