Impairment of mitochondrial anti-oxidant defence in SOD1-related motor neuron injury and amelioration by ebselen

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There is now compelling evidence of mitochondrial dysfunction in motor neuron disease (MND), but the molecular basis of these abnormalities is unknown. It is also unclear whether the observed mitochondrial dysfunction plays a central role in disease pathogenesis, and if so, whether its amelioration might present therapeutic opportunities. We adopted a candidate generation approach using proteomics to screen for changes in mitochondrial protein expression in a well-validated cell-culture model of superoxide dismutase 1 (SOD1) related familial MND (fMND). Changed proteins were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectroscopy. Protein candidates included apoptotic regulators, anti-oxidants and components of the electron transport chain. Confirmatory Western blotting was performed, and validated protein expression changes were further investigated. Peroxiredoxin 3 (Prx3), a mitochondrial thioredoxin-dependent hydroperoxidase, is downregulated in the presence of mutant SOD1 in both our cell-culture model and in the spinal cord mitochondria of mutant SOD1 transgenic mice. We confirm the expression of Prx3 within the mitochondria of spinal motor neurons in mouse and humans by immunohistochemistry. Using quantitative real-time PCR (Q-PCR), we show that Prx3 is also downregulated in spinal motor neurons from patients with both sporadic (sMND) and SOD1-related fMND. In a disease characterized by oxidative stress, this represents a potentially important deficit in mitochondrial anti-oxidant defence. Recent evidence suggests that oxidative stress from aberrant copper chemistry may not play a major part in the pathogenesis of SOD1-related fMND. From the results of this study we propose disruption of mitochondrial anti-oxidant defence as an alternative mechanism whereby mutant SOD1 may generate oxidative stress within motor neurons. We further demonstrate that ebselen, an anti-oxidant drug already safely used in human studies and that acts as a Prx mimic, is able to ameliorate the toxicity of mutant SOD1 in our cell-culture model. We conclude by showing that ebselen is capable of inducing transcription of the anti-oxidant response element (ARE) and postulate that ebselen may act both by the transcriptional upregulation of anti-oxidant proteins, and directly as an anti-oxidant in its own right.

Keywords: motor neuron disease; mitochondria; peroxiredoxin 3; ebselen; proteomics

Abbreviations: APRT = adenyl phosphoribosyl transferase; ARE = antioxidant response element; CHO = Chinese hamster ovary; fMND = familial MND; Gal = galectin; MALDI–TOF = matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MND = motor neuron disease; mSOD1 = murine SOD1; NTG = non-transgenic; Prx = peroxiredoxin; Q-PCR = quantitative real-time polymerase chain reaction; SEM = standard error of the mean; sMND = sporadic MND; SOD1 = superoxide dismutase 1; WT = wild-type

Introduction

Motor neuron disease (MND) is an adult-onset neurodegenerative disease dominated by the progressive dysfunction and death of motor neurons. Although most MND occurs sporadically, 2% is caused by mutations to Cu/Zn superoxide dismutase 1 (SOD1) (Rosen et al., 1993). It remains uncertain how >100 different mutations across all exons of this ubiquitously expressed free radical scavenging enzyme cause the death of motor neurons, but it is clear that mutant SOD1 exerts its effects via a toxic gain of function. There are several competing pathogenetic hypotheses, which are unlikely to be mutually exclusive. These now include good evidence of mitochondrial dysfunction in both sporadic (sMND) and familial MND (fMND) (Wood-Allum and Shaw, 2003).

Mitochondrial abnormalities of mitochondria have been documented in the synaptic terminals of spinal motor neurons of MND patients (Siklos et al., 1996). Histological studies of G93A SOD1 transgenic mouse spinal cord reveal dramatic morphological abnormalities of mitochondria at symptom onset shared by some, but not all, SOD1 transgenic mouse lines (Wong et al., 1995; Bruijn et al., 1997; Kong and Xu, 1998). Mitochondrial morphological and functional abnormalities are seen in motor neuronal cell lines expressing G93A human SOD1 (Menzies et al., 2002). Changes in the activities of complexes of the electron transport chain have also been shown in the brain and spinal cord of MND patients (Fujita et al., 1996; Borthwick et al., 1999) and in G93A transgenic mice (Mattiazzi et al., 2002). Alterations in mitochondrial calcium-handling are seen (Curti et al., 1996; Swerdlow et al., 1998), and there is evidence of the oxidative modification of mitochondrial macromolecules (Dhaliwal and Grewal, 2000; Mattiazzi et al., 2002). Although SOD1 was initially described as an exclusively cytoplasmic protein, it now appears that SOD1 is also present within the intermembrane space of mitochondria. The identification of the disease-causing mutant protein within mitochondria (Mattiazzi et al., 2002; Vijayvergiya et al., 2005) informed our decision to study changes in mitochondrial protein expression and justifies the wider interest in mitochondrial pathology in MND.

NSC34 cells were generated by fusion of a murine embryonic spinal cord culture enriched for motor neurons and a neuroblastoma cell line and, although immortalized, they retain key characteristics of motor neurons (Cashman et al., 1992; Durham et al., 1993). Use of this cellular model allows motor neuronal cells to be studied uncontaminated by other cells, unlike work done in transgenic mouse spinal cord. Previously published findings in these cells have been corroborated by findings in transgenic mice and in MND patients (Allen et al., 2003; Kirby et al., 2005).

It is uncertain whether the amelioration of mitochondrial dysfunction might be beneficial or even whether mitochondrial dysfunction plays a direct role in disease pathogenesis. Compounds believed to improve the bioenergetics of mitochondria are encouraging in their effects in SOD1 transgenic mice (Matthews et al., 1998; Klivenyi et al., 1999; Ferrante et al., 2001; Kira et al., 2006), but so far only creatine has been tested in patients and with negative results (Mazzini et al., 2001; Shefner et al., 2004). If the amelioration of mitochondrial dysfunction in MND is to be rationally attempted, its molecular basis must first be better understood.

The aims of this study were therefore (i) to identify changes in mitochondrial protein expression brought about by mutant SOD1 in NSC34 cells; (ii) to investigate key protein changes in post-mortem material from MND patients; and (iii) to investigate whether the toxicity of mutant SOD1 could be mitigated by modulation of these protein changes. We adopted a hypothesis-generating approach, first using proteomics to screen mitochondrially enriched fractions of NSC34 cells for protein expression changes brought about by the presence of mutant SOD1. This initial screen was carried out in single-cell clones of NSC34 cells expressing wild-type human SOD1 (WT cells), G93A mutant human SOD1 (G93A cells) and empty transfection vector (empty-vector cells). Changed proteins were identified by mass spectrometry and identifications were independently confirmed by Western blotting. As our initial screening experiment was carried out in single-cell clones, it was necessary to exclude the possibility of clone-specific protein changes. We therefore validated protein changes of particular interest by further Western blotting in (i) a second G93A single-cell clone; (ii) NSC34 cells expressing G37R mutant SOD1; or (iii) mitochondrial preparations of spinal cord from SOD1 transgenic mice. Subsequent work was confined to protein expression changes thus validated.

Material and methods

General laboratory reagents were from Sigma (Poole, UK). Tissue culture reagents were from Invitrogen (Paisley, UK).

NSC34 cell culture

Monoclonal NSC34 cells stably transfected with the pCEP4 empty transfection vector (empty-vector cells), wild-type human SOD1 (WT cells), G93A mutant human SOD1 (G93A cells) and G37R mutant human SOD1 (G37R cells) were used and cultured as described previously (Menzies et al., 2002).

SOD1 transgenic mice

G93A SOD1 mice and age-matched wild-type (WT) SOD1 and non-transgenic (NTG) mice were compared. Mice were killed at 30, 60 and 90 days. Mice bred on a C57BL6 background (Dal Canto and Gurney, 1994; Gurney et al., 1994) were originally purchased from The Jackson Laboratory (Bar Harbor, Maine).

Mitochondrial preparations

Mitochondrially enriched fractions of NSC34 cells were prepared as described previously (Menzies et al., 2002). Murine spinal cords were homogenized on ice in 10 volumes of homogenization buffer (0.25 M sucrose, 10 mM TEA, 50 mM KCl, pH 7.8 (acetate acid),...
1x mini EDTA-free protein inhibitor cocktail tablet per 10 ml. Homogenate was spun at 1000 g for 10 min and the supernatant (S1) was spun at 17 000 g for 20 min, generating a P2 pellet (mitochondrially enriched fraction) and a further (S2) supernatant. The P2 pellet was re-suspended in 300 µl of homogenization buffer. Two hundred and seventy microlitres of this suspension was then added to 1.98 ml of 5 volumes of 50% OptiPrep™ (Axis-Shield, Oslo, Sweden) and 1 volume of dilution buffer (0.25 M sucrose, 6 mM EDTA, 60 mM HEPES, pH 7.4). The sample was overlain sequentially by 0.75 ml of 25% Optiprep and then 0.75 ml of 20% Optiprep, and the ultracentrifuge tube was topped up with dilution buffer. The tube was spun at 170 000 g for 3 h at 4°C. Samples were removed from the 44/25% (I3), 25/20% (I2) and 20/0% (I1) interfaces. The I2 fraction contained the mitochondria. Proportional fractions of this preparation were Western blotted for markers of mitochondria, cytosol and endoplasmic reticulum to assess mitochondrial purity (Fig. 5D).

**Mitochondrial pellet rehydration**

**2D gel electrophoresis**

Mitochondrially enriched pellets of NSC34 cells (G93A, WT SOD1 or empty-vector) were rehydrated in mitochondrial solubilization buffer (MSB) (Rabilloud et al., 1998). Protein concentrations were measured after an hour using a modified Bradford assay (Pierce, Rockford, IL, USA).

**1D electrophoresis**

Mitochondrially enriched pellets of NSC34 cells were rehydrated in extract buffer [20 mM HEPES/KOH, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM dithiothreitol (DTT), 2 mM sucrose, Complete EDTA-free™ protease inhibitor mixture (Roche, Basel, Switzerland)—1 tablet per 10 ml] before sonication. Protein concentrations were measured as before. Samples for Western blotting were made up in 2x sample buffer (100 mM Tris, pH 6.8, 4% w/v SDS, trace bromophenol blue, 20% v/v glycerol). The protein concentrations of mitochondrially enriched pellets (P2) from mouse spinal cords were measured as above and samples were made up in 2x sample buffer.

**2D SDS–PAGE**

Bio-Rad (Hercules, CA, USA) 24-cm immobilized pH gradient strips (IPG strips) with a pH gradient of 3–10 were loaded with IPG strips (IPG strips) with a pH gradient of 3–10 were loaded with 2D gels

Where a band could not be seen on a 1D gel loaded with 10 µg of protein, 2D gels loaded with 100 µg of protein were run. A Coomassie-stained gel was run alongside in order that the area of each gel containing the spot of interest could be excised and transferred to PVDF (Immobilon™-P transfer membrane, Millipore, Billerica, MA, USA).

**MALDI–TOF MS**

Preparative 2D gels were run and stained using Bio-Safe™ Coomassie (Bio-Rad). Spots of interest were excised and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF MS) at the Aberdeen Proteomics Facility, UK. An Investigator ProtGest workstation (Genomic Solutions, Huntingdon, UK) was used to perform in-gel trypsin digestion. MALDI–TOF MS was then performed on a Voyager-DE STR instrument (Perspective Biosystems, Framingham, MA, USA). Software from Mascot (Matrix Science, London, UK) and MS-Fit (University of California, San Francisco, CA, USA) was used to interrogate the NCBI (ncbi.nlm.nih.gov) and SwissProt/TrEMBL databases (www.ca.expasy.org) in order to obtain a match and identify each protein spot. Independent confirmation of protein identification and expression change was sought by Western blotting.

**Confirmatory Western blotting**

**1D gels**

Western blotting was performed as described previously (Menzies et al., 2002) in at least two further mitochondrial preparations. Prohibitin, heat shock protein 60 (HSP60) or cytochrome oxidase subunit 4 (COX4) was used as loading control.
genic mice were treated as above (anti-Prx3, 1:3000).

paraformaldehyde-perfused 90 day NTG and G93A SOD1 transgenic mice, 
biotinylated markers were used ExtrAvidin mouse, 1:1000 (DAKO); rabbit anti-goat, 1:2000 (DAKO). Where necessary antibodies were HRP-conjugated and applied for 1 h at room temperature. Swine anti-rabbit, 1:2000 (DAKO, Glostrup, Denmark); goat anti-sheep, 1:1000 (Sigma, Poole, UK), goat anti-mouse, 1:1000 (DAKO), rabbit anti-goat, 1:2000 (DAKO). Where biotinylated markers were used ExtrAvidin™ (Sigma), 1:5000 was added to the secondary antibody. Western blots were quantified by densitometry in a MultiImage™ Light cabinet using Alphalnnotech software (Flowgen Bioscience Ltd, Nottingham, UK).

Secondary antibodies were added to the secondary antibody. Western blots were quantified by densitometry in a MultiImage™ Light cabinet using Alphalnnotech software (Flowgen Bioscience Ltd, Nottingham, UK).

Immunocytochemistry
NSC34 cells were grown on poly l-lysine-coated coverslips, fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100 and blocked in 5% normal horse serum. Primary antibodies were mouse anti-HSP60, 1:500 (Abcam) and rabbit anti-Prx3, 1:2000 (LabFrontier). Secondary antibodies were anti-mouse-AlexaFluor488 and anti-rabbit-AlexaFluor555, 1:1000 (Molecular Probes).

Ethical approval for use of human material
Post-mortem tissue for immunohistochemistry and quantitative real-time PCR (Q-PCR) was collected by the Newcastle (UK) and Sheffield (UK) brain tissue banks using protocols approved by the local ethics committees and in compliance with MRC guidelines (http://www.mrc.ac.uk/pdf-nervous_tissue_guidance.pdf). Informed consent was obtained.

Immunohistochemistry
Human spinal cord was fixed and embedded in paraffin, and 5 μm serial sections were made. Immunohistochemistry was performed as described previously (Allen et al., 2003) using anti-Prx3 (LabFrontier), 1:2000 for 1 h at room temperature. Peroxiredoxin (Prx3) staining of lumbar spinal cord of two SOD1-related fMND cases and two age- and sex-matched neuropathologically normal controls was compared. Post-mortem delays were comparable. Immunohistochemistry was performed as described previously (Allen et al., 2003) using anti-Prx3 (LabFrontier), 1:2000 for 1 h at room temperature. Peroxiredoxin (Prx3) staining of lumbar spinal cord of two SOD1-related fMND cases and two age- and sex-matched neuropathologically normal controls was compared. Post-mortem delays were comparable.

Quantitative PCR
Lumbar spinal cord blocks were snap-frozen in liquid nitrogen surrounded by melting Arcton (ICI Performance Chemicals, Runcorn, UK). Sections of 10 μm were made in a cryostat (Bright Instrument Co. UK, Huntingdon, UK) and thaw-mounted onto Super frost charged slides. Slides were defrosted before rapid fixation in 70% ethanol for 30 s. After rinsing in diethylylpropylcarbodi-methylimidazole (DEPC) treated water, slides were stained for 1 min with toluidine blue. After a further rinse, slides were progressively dehydrated in 70, 95 and 100% ethanol. Slides were immersed in xylene (2 x 5 min) before air-drying. An Arcturus Pixcell II laser capture microdissector (Mountain View, CA, USA) was used to isolate individual motor neurons from lumbar spinal cord sections from three neuropathologically normal controls, three sMND cases and three SOD1 fMND cases as described previously (Heath et al., 2002). Mean post-mortem delays were comparable. Approximately 1000 motor neurons were isolated per case. Following RNA extraction using PicoPure RNA Isolation Kit (Arcturus), a double round of RNA amplification was performed using the RiboAmp RNA Amplification kit (Arcturus), according to the manufacturer’s protocol.

Table 1 Primer design and optimization for Q-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Optimum concentration (pM)</th>
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</thead>
<tbody>
<tr>
<td>Actin 5’ forward</td>
<td>ATC CCC CAA AGT</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>TCA CAA TG</td>
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<tr>
<td>Actin 5’ reverse</td>
<td>GTG GCT TTT AGG</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>ATG GCA AG</td>
<td></td>
</tr>
<tr>
<td>Prx3 forward</td>
<td>TTA AAC ATG GTT AGT</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>TGC TAG TAC AAG GA</td>
<td></td>
</tr>
<tr>
<td>Prx3 reverse</td>
<td>TTG AGA CAT GAT CTA</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>AGA ATA GCC TTC TA</td>
<td></td>
</tr>
</tbody>
</table>

Neuroprotective effect of ebselen
NSC34 cells were grown in 96-well plates in Dulbecco-modified essential medium (DMEM) + 10% fetal calf serum (FCS) until ~60% confluent. Medium was replaced with fresh DMEM ± 10% serum ± ebselen (C13H9NOSe) (Sigma) at a concentration of 1, 5, 10 or 20 μM. Ebselen was dissolved in dimethyl sulfoxide (DMSO). MTT assays were performed as described previously (Cookson et al., 1998).

Anti-oxidant response element (ARE) reporter assay
Chinese hamster ovary (CHO) cells were cultured in DMEM supplemented with 10% FCS and penicillin/streptomycin. ARE-TK-GFP and TK-GFP reporter constructs were donated by William...
The TK-EGFP reporter construct is a 123-bp thymidine kinase promoter inserted into the multiple cloning site of pEGFP (Clontech, Mountain View, CA, USA). The ARE-TK-EGFP construct additionally contains four repeats of a 41-bp GST ARE motif, 3′ to the TK promoter (Zhu and Fahl, 2001) (TAGCTTGGAAATGACATTGCTAATCGTGACAAACTTT). Plasmids were transfected into CHO cells using Lipofectamine 2000 (Invitrogen). After 10–14 days selection in 0.5 mg/ml G418, cells were expanded and selected for basal eGFP expression using fluorescence-activated cell sorting (BD, FACS Aria) with two sequential cell sorts for each cell line. Mixed populations of stable transfectants with basal eGFP expression were subsequently used and designated 4xARE-TK-GFP CHO (ARE-containing line) and TK-GFP CHO (control cell line). Cells were plated in standard medium in 96-well plates at 5 × 10^4 cells per well in triplicate for each assay point. Medium was removed at 24 h and replaced with FCS-free medium containing 0.1% DMSO (vehicle) or vehicle plus ebselen at concentrations of 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 μM. Plates were incubated for 24 h and fluorescence was measured on a plate reader using 485/530 nm excitation/emission filters. Non-linear regression was used to fit a sigmoidal dose–response curve on a semi-log plot and to calculate an EC50 using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

### Results

#### Proteomic screening experiment

Approximately 1200 protein spots were seen on 2D gels made from mitochondrially enriched NSC34 cell fractions. Twenty-nine spots changed in volume in a mutant-specific fashion, of which 18 were increased and 11 were decreased. Of these 29 spots, 24 positive MALDI–TOF identifications were made (Fig. 1) on the basis of percentage sequence coverage, molecular weight search (MOWSE) score and the calculated molecular weight (MW) and pI of the candidate protein compared with those of the spot on the 2D gels (Tables 2 and 3). Table 4 groups candidate proteins of interest by function.

#### Confirmation of protein identifications

When multiple factors are taken into account as described above, it is possible, confidently, to identify most protein spots using MALDI–TOF MS. All such protein identifications are unlikely to be unambiguous; therefore, confirmatory Western blotting was performed in the same G93A, WT

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**Fig. 1** Representative 2D gel of a mitochondrially enriched fraction of NSC34 cells. Approximately 1200 individual protein spots appear on the gels. Protein spots whose volume changed in a mutant SOD1-specific fashion that were successfully identified by MALDI–TOF MS are identified. G93A versus empty-vector n = 7 gel pairs; WT versus empty-vector n = 8 gel pairs. Spot numbering refers to Tables 2 and 3.
Table 2  Protein expression changes in the presence of G93A mutant human SOD1

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein</th>
<th>Swiss Prot or NCBI accession no.</th>
<th>G93A versus NTG Mean fold change</th>
<th>Wilcoxon P-value</th>
<th>WTSOD1 versus NTG Mean fold change</th>
<th>Wilcoxon P-value</th>
</tr>
</thead>
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<tr>
<td>217</td>
<td>Aldehyde dehydrogenase 2 (mitochondrial)</td>
<td>P477738</td>
<td>↓ 1.7 0.03</td>
<td>← NA 0.08</td>
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<td></td>
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<tr>
<td>227</td>
<td>Aldehyde dehydrogenase 7 family member A1</td>
<td>Q9DBF1</td>
<td>↑ 1.9 0.03</td>
<td>← NA 1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>304</td>
<td>Mitochondrial acyl-CoA thioesterase-1 (MTE-1)</td>
<td>Q9QYR9</td>
<td>↑ 1.8 0.02</td>
<td>← NA 0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>344</td>
<td>Arsenite transporter (ARS1A)</td>
<td>O54984</td>
<td>↑ 3.2 0.02</td>
<td>↓ 1.9 0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>372</td>
<td>3-hydroxyisobutyryl-coenzyme A hydrolase</td>
<td>Q8QZ51</td>
<td>↑ 1.5 0.03</td>
<td>← NA 0.20</td>
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<tr>
<td>373</td>
<td>Lysosomal ATPase H⁺ transporter</td>
<td>g31981304</td>
<td>↑ 1.6 0.03</td>
<td>↓ 1.6 0.01</td>
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<tr>
<td>426</td>
<td>Hexaprenyldihydroxybenzoate methyltransferase, mitochondrial precursor</td>
<td>g26330600</td>
<td>↑ 2.6 0.02</td>
<td>← NA 0.15</td>
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<tr>
<td>482</td>
<td>Halacid dehalogenase-like hydrolase domain containing 3</td>
<td>Q9CYW4</td>
<td>↑ 2.4 0.02</td>
<td>← NA 0.74</td>
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<tr>
<td>513</td>
<td>Ubiquitin carboxyl-terminal hydrolase L1 (UBCH 1)</td>
<td>Q9R0P9</td>
<td>↑ 1.5 0.02</td>
<td>← 1.5 0.01</td>
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<tr>
<td>515</td>
<td>Peroxiredoxin</td>
<td>O88007</td>
<td>↑ 1.4 0.03</td>
<td>↑ 1.2 0.01</td>
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<tr>
<td>530</td>
<td>Peroxiredoxin</td>
<td>P20108</td>
<td>↑ 3.0 0.02</td>
<td>← NA 0.15</td>
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<tr>
<td>531</td>
<td>NADH dehydrogenase (ubiquinone) flavoprotein 2</td>
<td>Q9D6J6</td>
<td>↑ 1.1 0.02</td>
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<td>539</td>
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<td>557</td>
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<td>Q6117</td>
<td>↑ 1.6 0.02</td>
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<td>565</td>
<td>Peptidyl-tRNA hydrolase 2, mitochondrial precursor</td>
<td>Q8R2YB8</td>
<td>↑ 1.5 0.02</td>
<td>← NA 0.31</td>
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<tr>
<td>570</td>
<td>Second mitochondria-derived activator of caspases</td>
<td>Q9JLQ3</td>
<td>↑ 1.6 0.02</td>
<td>≤ 2.6 0.01</td>
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<td>571</td>
<td>Cytochrome b5 outer mitochondrial membrane precursor</td>
<td>O43169</td>
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<td>≤ 1.3 0.04</td>
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<td>585</td>
<td>Human superoxide dismutase I</td>
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<td>≤ 1.3 0.01</td>
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<tr>
<td>622</td>
<td>Mouse superoxide dismutase I</td>
<td>P08228</td>
<td>↑ 1.8 0.02</td>
<td>≤ 1.4 0.01</td>
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<td>645</td>
<td>Galectin I</td>
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<td>← NA 0.38</td>
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<td>713</td>
<td>Riken cDNA 231005014</td>
<td>g20885971</td>
<td>↑ 1.6 0.02</td>
<td>← NA 0.31</td>
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<td>1046</td>
<td>Cytochrome c₁</td>
<td>Q9D0M3</td>
<td>↑ 2.3 0.02</td>
<td>← NA 0.31</td>
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<tr>
<td>1052</td>
<td>Cytochrome c₁</td>
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<td>← NA 0.20</td>
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<tr>
<td>1272</td>
<td>Adeny1 phosphoribosyl transferase</td>
<td>P08030</td>
<td>↑ 4.0 0.02</td>
<td>≤ 1.5 0.01</td>
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</table>

↓ = decreased expression; ↑ = increased expression; ← = unchanged expression.

Spot 585 was present only on G93A gels. It was not visible on WT SOD1 or empty-vector gels.

and empty-vector single-cell clones used in the proteomic screening experiment. Western blotting of mitochondrially enriched fractions of NSC34 cells was performed for those proteins for which antibodies were available (Figs 2 and 3). Any alteration in mitochondrial matrix mass induced by mutant SOD1 was controlled for by concomitant blotting for prohibitin, a mitochondrial matrix protein, and expressing protein expression relative to that of prohibitin.

Reductions in Prx3 (Fig. 2A), Prx4 (Fig. 3A) and mitochondrial acyl-CoA thioesterase-1 (MTE1) (Fig. 3B) in G93A cells were demonstrated, lending independent support to these MALDI–TOF MS protein identifications. The increase in Prx3 expression seen in WT cells was not statistically significant in the 2D experiment.

Validated reexpression of Prx2 (Fig. 2B), the second mitochondrial activator of caspases (SMAC) (Fig. 2C), endogenous murine SOD1 (mSOD1) (Figs 2D and 3C), galectin 1 (Gal 1) (Fig. 2E), cytochrome c₁ (Fig. 2F) and adenyl phosphoribosyltransferase 1 (APRT1) (Fig. 2G) in G93A cells was also shown, supporting these MALDI–TOF MS protein identifications. The reductions in mSOD1, SMAC, APRT1 and Gal 1 in WT cells seen in the 2D experiment were confirmed. The reduction in Prx2 in WT cells was not confirmed. The Prx4, (MTE1), mSOD1, Prx2 and Prx3 immuno-reactive spots appearing on blots of 2D gels co-localized with their respective protein spots on Coomassie-stained gels run in parallel, lending further support to these protein identifications (Prx2 and 3 and 3 blots not shown).

Spot 585 appeared only on G93A gels and could not be identified by MALDI–TOF MS. Western blotting of 2D gels, however, indicated that transgenic human SOD1 co-localized with Spot 585 (Fig. 3C). Western blotting for SOD1 of 1D gels confirmed that both G93A and G37R mutant human SOD1 were present in the relevant mitochondrial enriched fractions of G37R cells confirmed its downregulation in WT SOD1 and mSOD1, albeit in much smaller quantities.

Validation of protein spots of interest

To exclude the possibility of clonal effects in confirmed protein changes of particular interest, we undertook further Western blotting in one or more of (i) another G93A-expressing single-cell clone; (ii) NSC34 cells expressing G37R mutant human SOD1; (iii) mitochondrial preparations of whole spinal cord from G93A transgenic mice.

Peroxiredoxin 3

Western blotting for Prx3 in mitochondrially enriched fractions of G37R cells confirmed its downregulation in the presence of a second species of mutant SOD1 (Fig. 4).
Prx3 was also downregulated in another G93A single-cell clone (data not shown). Western blotting of mitochondrially enriched (P2) fractions of whole spinal cord from NTG, WT and G93A mice at 90 days, moreover, confirmed a decrease in Prx3 expression in the presence of G93A SOD1 (Fig. 5A). The apparent increase in Prx3 expression seen in WT mice was not statistically significant. No change in Prx3 expression could be demonstrated in mitochondrially enriched fractions of whole spinal cord from 30 day or 60 day G93A mice.

Superoxide dismutase 1

Western blotting for SOD1 of mitochondrially enriched fractions of G93A cells confirmed that both G93A mutant human SOD1 and endogenous mSOD1 were present. Blotting of mitochondrially enriched fractions of WT cells confirmed that both WT human SOD1 and mSOD1 were present. Blotting of another G93A-expressing single-cell clone for SOD1 did not show an increase in mSOD1 expression, nor was an increase evident in G37R cells. Given the controversy in the field regarding which forms of SOD1 gain access to mitochondria and the multiple-fold over-expression of G93A mutant SOD1 in our strain of transgenic mice, we were, nevertheless, interested to see whether we could demonstrate mSOD1 upregulation in the mitochondria of G93A transgenic mouse spinal cord. Gradient-purified mitochondria and mitochondrially enriched (P2) fractions from mouse spinal cord were blotted for SOD1 (Fig. 5B and C). G93A SOD1 was present within purified mitochondria in quantity. Endogenous mSOD1 was also present. WT human SOD1 was present in the spinal cord mitochondria of mice over-expressing normal human SOD1. Higher MW bands, most likely SOD1 dimers, were seen in P2 fractions of WT and G93A mice. In gradient-purified mitochondria from G93A mice, mSOD1 expression was increased when compared with that in WT and NTG mice (Fig. 3C).

Table 3  Identification of protein spots whose spot volume changes in the presence of G93A mutant SOD1

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein identity</th>
<th>SwissProt or NCBI accession no.</th>
<th>Predicted MW (kDa)*</th>
<th>Measured gel MW (kDa)</th>
<th>Theoretical pI</th>
<th>Estimated gel pI</th>
<th>Sequence coverage %</th>
<th>MOWSE score</th>
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<tr>
<td>217</td>
<td>Aldehyde dehydrogenase 2 (mitochondrial)</td>
<td>P47738 54 374.87 47 900 6.05 5.0–6.0 38 7.732·107</td>
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<tr>
<td>227</td>
<td>Aldehyde dehydrogenase 7 family member A1</td>
<td>Q9DBF1 55 645 48 700 6.0 5.0–6.0 34 5.837·106</td>
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<td>304</td>
<td>Mitochondrial acyl-CoA thioesterase-1 (MTE-1)</td>
<td>Q9QYR9 44 933.54 43 200 6.14 5.0–6.0 32 6.138·104</td>
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<td>344</td>
<td>Arsenite transporter (ARSA1)</td>
<td>O54984 38 823 40 600 4.8 4.5 40 4.672·108</td>
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<td>3-Hydroxyisobutyryl-coenzyme A hydrolase</td>
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<td>Lysoosomal ATPase H+ transporter</td>
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<td>426</td>
<td>Hexaprenyldihydroxybenzoate methyltransferase, mitochondrial precursor</td>
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<td>482</td>
<td>Halocacid dehalogenase-like hydrolase domain containing 3</td>
<td>Q9CYW4 28 027 30 600 6.3 5.0–6.0 32 3472</td>
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<td>513</td>
<td>Ubiquitin carboxy-terminal hydrolase L1 (UBCH 1)</td>
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<td>515</td>
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<tr>
<td>530</td>
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<td>P20108 21 564.58 26 800 5.31 5.0–6.0 53 8100</td>
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<td>531</td>
<td>NADH dehydrogenase (ubiquinone) flavoprotein 2</td>
<td>Q9D6J6 23 991 26 800 5.31 5.0–6.0 53 8100</td>
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<td>539</td>
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<td>Peptidyl-tRNA hydrolase 2, mitochondrial precursor</td>
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<td>570</td>
<td>Second mitochondria-derived activator of caspases</td>
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<td>571</td>
<td>Cytochrome b5 outer mitochondrial membrane precursor</td>
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<td>622</td>
<td>Mouse superoxide dismutase 1</td>
<td>P08228 15 943 18 600 6.0 5.0–6.0 42 835</td>
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<td>1046</td>
<td>Cytochrome c, mitochondrial</td>
<td>Q9DOM3 32 966.75 31 900 7.12 5.0–6.0 29 2.372·104</td>
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<td>1052</td>
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<td>1272</td>
<td>Adenyl phosphoribosyl transferase 1</td>
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*Predicted molecular masses given with mitochondrial leader sequences removed where relevant.
Table 4  The 24 of 29 changed proteins identified by MALDI–TOF MS arranged by function

<table>
<thead>
<tr>
<th>Anti-oxidant proteins</th>
<th>Electron carriers</th>
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<tr>
<td>Peroxiredoxin</td>
<td>Cytochrome c₁↑</td>
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<td>Peroxiredoxin</td>
<td>Cytochrome MOMb5↑</td>
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<td>Peroxiredoxin</td>
<td>NADH dehydrogenase flavoprotein 2↑</td>
</tr>
<tr>
<td>Murine SOD1↑</td>
<td>Hexaprenyldihydroxycvelte methyltransferase↓</td>
</tr>
<tr>
<td>Human SOD1↑</td>
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</table>

| Apoptosis                   | Metabolic enzymes                |
| Arsinite transporter↑       | 3-Hydroxyisobutyryl-coenzyme A hydrolase↓ |
| Galectin I↑                 | Aldehyde dehydrogenase family 7 member 1↓ |
| Second mitochondria-derived activator of caspases↑ | Aldehyde dehydrogenase 2 (mitochondrial) ↓ Mitochondrial acyl-CoA cholesterol esterase I (MTE-I)↓ |

| Protein processing           | Miscellaneous                    |
| Ubiquitin carboxyl-terminal hydrolase I↑ | Lysosomal ATPase H+ transporter↑ |
| Galectin I↑                  | Riken cDNA 2310005014↑           |
| Peptidyl-tRNA hydrolase 2 (mitochondrial precursor)↓ | Adeny1 phosphoribosyl transferase I↑ Haloacid dehydrogenase-like hydrolase domain containing 3↓ |

Two changed protein spots (spots 1046 and 1052) were both identified as cytochrome c₁. The arrow indicates the direction of protein expression change in the presence of G93A mutant human SOD1.

Fig. 2 Confirmatory 1D Western blots of mitochondrially enriched fractions of NSC34 cells. (A) Prx3, (B) Prx2, (C) SMAC, (D) endogenous mSOD1, (E) Gal 1, (F) Cytochrome c₁, (G) APRT 1. Prohibitin (H), HSP60 or COX4 were used as loading controls. (I–M) Quantification of Western blotting. Data are from 2–5 replicates in at least two further mitochondrial preparations. Columns show mean OD corrected for loading (test OD/control OD). Columns marked with an asterisk (*) differ statistically significantly from the vector (P < 0.05 Wilcoxon matched-pairs test). Error bars are 1 SEM. (I) Prx3, (J) Prx2, (K) SMAC, (L) mSOD1, (M) Gal 1.
**Peroxiredoxin 2**

Western blots for Prx2 in G37R cells confirmed its upregulation in the presence of a second species of mutant SOD1 (Fig. 4). This change was not demonstrated in mitochondrial preparations of whole spinal cord from G93A mice.

**Galectin 1**

Western blots for Gal 1 of mitochondrially enriched fractions of two G93A-expressing NSC34 single-cell clones confirmed the upregulation of this protein in the 2D experiment (data not shown). There was no Gal 1 upregulation in G37R cells.

**Prx3 localization**

Prx3 immunocytochemistry in NSC34 cells generated a granular staining pattern that co-localized with staining for HSP60, a mitochondrial matrix protein, confirming the mitochondrial localization of Prx3 (Fig. 6A–D). Prx3 immunohistochemistry in lumbar spinal cord from SOD1 mice and human lumbar spinal cord, moreover, confirmed Prx3 expression within the mitochondria of spinal motor neurons (Fig. 6E–J). Comparison of Prx3 staining of spinal motor neurons from SOD1-related fMND cases and normal controls revealed greater variability of staining between individual motor neurons in SOD1-related fMND cases than in controls, which were uniformly strongly stained (Fig. 6E). In SOD1-related fMND cases, degenerative motor neurons were found with only faint Prx3 staining (Fig. 6F). fMND motoneurons were also seen with normal staining, however, perhaps representing motor neurons at an earlier stage in the disease process.

**Q-PCR in human spinal motor neurons**

Prx3 Q-PCR was performed in motor neurons picked by laser capture microdissection from sections of lumbar spinal cord from three neuropathologically normal controls, three sMND and three SOD1-related fMND cases. The data from triplicate experiments indicate that Prx3 is expressed at lower levels in both sMND and SOD1-related fMND cases than it is in neuropathologically normal controls (Fig. 7).

**Neuroprotective effect of ebselen**

Serum withdrawal induced cell death in all NSC34 cell lines examined. This cell death was most marked in G93A and G37R cells (Fig. 8): NTG cells mean survival 48 h post-serum withdrawal = 62.72% $\pm$ 2.10, 1 standard error of the mean (SEM); WT cells = 57.03% $\pm$ 5.57, 1 SEM; G93A cells = 32.85% $\pm$ 2.28, 1 SEM; G37R cells = 39.13% $\pm$ 3.90, 1 SEM). Differences in cell death between both mutant and both control cell lines were statistically significant.
Death did not significantly differ between NTG and WT cells ($P = 0.37$) or between G93A and G37R cells ($P = 0.34$). Application of ebselen 1, 5, 10 or 20 μM at the time of serum withdrawal had no significant effect on cell death in NTG cells or WT cells, but reduced cell death in G93A and G37R cells (Fig. 8A–D). This mutant-specific rescue was dose-dependent. Ebselen 1 μM did not rescue any cell line assayed. Ebselen 5 μM maximally increased G93A cell survival from a mean of 32.85% ($\pm$ 2.04, 1 SEM) to a mean of 44.34% ($\pm$ 1.37, 1 SEM), an increase of 35%. Ebselen 10 μM maximally increased G37R cell survival to a mean of 67.21% ($\pm$ 3.76, 1 SEM), an increase of 71.8%. A degree of high-dose toxicity was evident in G93A cells but not G37R cells. There was no rescue of any cell line with DMSO (data not shown). Data were the results of triplicate wells in three independent experiments. A further set of experiments in a second single-cell clone of WT and G93A cells was carried out. As previously, the G93A cells died more than the WT cells on serum withdrawal, and there was selective and statistically significant rescue by ebselen of the G93A cells alone (data not shown).

**Induction of the ARE by ebselen**

Ebselen induced transcription of the ARE in CHO cells transfected with the ARE-TK-EGFP reporter construct, but not in those transfected with the control TK-GFP construct (Fig. 9). Three independent repeats of the experiment were performed with three replicates per condition. The mean EC$_{50}$ at 24 h of these three experiments was 5.3 μM ± 0.34 (±1 SEM).

**Discussion**

We employed a candidate-generating approach, first using proteomics to identify mitochondrial proteins whose expression changed in the presence of mutant SOD1 in a...
well-validated model of SOD1-related fMND. Proteins whose expression changed in a mutant-specific fashion had functions potentially relevant to the pathogenesis of MND including roles in apoptosis, protein processing and anti-oxidant defence. We then validated selected protein changes of interest by Western blotting in mitochondrial preparations of further NSC34 cells and G93A transgenic mouse spinal cord. Prx3, a thioredoxin-dependent hydroperoxidase (Fig. 10A), was downregulated in NSC34 cells expressing two different species of mutant SOD1 and in 90 day G93A transgenic mouse whole spinal cord. Prx3 immunohistochemistry confirmed its expression within the mitochondria of human and murine spinal motor neurons. We then used Q-PCR to show a downregulation

Fig. 6 Localization of Prx3. (A–D) Immunocytochemistry of G93A NSC34 cells for Prx3 showed a granular pattern of staining. Staining for HSP60, a mitochondrial matrix marker, produced a similar pattern that co-localized with that of Prx3 confirming its mitochondrial localization. (A) Hoechst, (B) Prx3, (C) HSP60, (D) co-localization (scale bar = 10 μm). Prx3 immunostaining of human (E, F) and mouse (G–J) lumbar spinal cord motor neurons. The normal human tissue (E) and NTG mice (G and I) show a dense punctate pattern of staining that is identical to that observed using other markers of mitochondrial proteins. In contrast (F), a chromatolytic neuron from a patient with fMND due to the SOD1 I113T mutation shows a marked reduction in immunostaining of mitochondrial profiles (arrow = lipofuscin granules). In the G93A mouse model of SOD1-related fMND (H), much more variability in Prx3 staining of the remaining motor neurons is seen. At higher power (J), a vacuolated motor neuron (arrowhead) from a G93A mouse shows a reduced and abnormal distribution of staining. Images E, F, I and J captured using ×40 objective; images G and H captured using ×20 objective. Identical scaling performed for reproduction.
of Prx3 mRNA in spinal motor neurons from patients with both sporadic and SOD1-related fMND. We next demonstrated that the Prx-mimic ebselen (Fig. 10B) selectively protected motor neuronal cells expressing two different species of mutant human SOD1 against apoptosis induced by withdrawal of serum. This is the first demonstration of neuroprotection by ebselen in a model of MND. Finally, having postulated that ebselen may act by upregulating anti-oxidant protein transcription as well as act as an anti-oxidant in its own right, we show that ebselen induces transcription of the ARE, an oxidative stress-sensitive promoter sequence common to many anti-oxidant response genes, including members of the Prx family and the proteins that maintain them in their active, reduced state (Ishii et al., 2000; Shih et al., 2003).

A combination of two proteomic approaches was recently used helpfully to delineate the mitochondrial proteome of NSC34 cells (Fukada et al., 2004). On comparing mitochondrial protein expression in cells expressing mutant and WT human SOD1, altered post-translational modification of VDAC2 was highlighted. The NSC34 cells used, however, were a mixture of 10–20 clones (unlike our monoclonal cell lines), and the proteomic methodology differed. Confirmatory work in other MND models was not offered. SOD1 is no longer considered to be an exclusively cytoplasmic protein (Okado-Matsumoto and Fridovich, 2001; Sturtz et al., 2001; Higgins et al., 2002). In a careful study of G93A and WT transgenic mice, mutant human, WT human and endogenous mSOD1 were demonstrated in brain mitochondria (Mattiazzi et al., 2002). Liu et al. (2004) recently suggested that selective recruitment to spinal cord mitochondria of mutant SOD1 underlies their selective vulnerability and that WT human SOD1 and mSOD1 are excluded. This exclusion of WT human SOD1 from spinal cord mitochondria was, however, recently refuted (Vijayvergiya et al., 2005). We identified a protein upregulated in mitochondrially enriched fractions of G93A cells and downregulated in WT cells as mSOD1. In our hands, both mSOD1 and WT human SOD1 were readily detectable in gradient-purified mitochondria from 90 day WT SOD1 transgenic mouse spinal cord, albeit at lower levels than mutant SOD1 in spinal cord from G93A mice. Superoxide generated by the electron transport chain is released on both sides of the inner mitochondrial membrane (Han et al., 2001). As superoxide does not readily cross membranes, it must be dealt with where it is generated. Whilst manganese SOD is present in the mitochondrial matrix and converts superoxide to H₂O₂, it is not present within the intermembrane space, so it cannot reduce superoxide there. Superoxide released into the intermembrane space may instead be reduced by SOD1, thereby protecting that compartment from oxidative damage.

Mitochondria, which house the electron transport chain, are especially vulnerable to oxidative damage and are equipped with anti-oxidant defences. Dismutases reduce superoxide to H₂O₂, which is itself reduced to water by Prxs and glutathione peroxidases. The further reduction of H₂O₂ is important to prevent reaction with Fe³⁺ and the generation of the highly reactive hydroxyl anion. Catalase reduces H₂O₂ to water in other cellular compartments, but is absent from mitochondria (Radi et al., 1991). Prx3, moreover, is ~30 times more abundant within mitochondria than glutathione peroxidase-1 (Chang et al., 2004).

When rat hippocampus was injected with ibotenic acid, an acute oxidative insult, Prx3 mRNA was reduced by 50% at 6 h. Prior Prx3 over-expression inhibited post-insult gliosis and protein nitrosylation (Hattori et al., 2003). This suggested that acute oxidative injury may downregulate Prx3 and that Prx3 over-expression can protect against acute oxidative injury. That mutant SOD1 causes oxidative stress is evidenced by the progressive oxidative damage to lipids, proteins and nucleic acids seen in disease models and in SOD1-related fMND (Shaw et al., 1995; Hall et al., 1998; Smith et al., 1998; Liu et al., 1999). Our results indicate that this more chronic oxidative stress is associated with the downregulation of Prx3 and Prx4. Theoretically, this may occur at any stage from mRNA generation to post-translational processing. A microarray study recently performed in our NSC34 cells, however, independently identified a >4-fold reduction in Prx3 mRNA and a >2-fold reduction in Prx4 mRNA in the presence of G93A SOD1 (Kirby et al., 2005).

Prx2 was upregulated in mitochondrially enriched fractions of G93A and G37R cells. This increase may also be transcriptional as a 1.96-fold increase of Prx2 mRNA was found in G93A cells in our microarray study. There was no corresponding Prx2 upregulation in whole spinal cord

**Fig. 7** Q-PCR comparison of Prx3 mRNA levels in human spinal motor neurons picked from spinal cord sections taken from three cases each of sMND and SOD1-related fMND compared with neuropathologically normal controls. Prx3 expression in each case is normalized to the expression level of actin then shown relative to mean control case expression. There is less Prx3 mRNA present in spinal motor neurons from patients with either the sporadic or the SOD1 familial form of the disease than there is in the spinal motor neurons of neuropathologically normal controls (*P < 0.05 versus normals).
Mitochondrial anti-oxidant defence in MND

Fig. 8 Effect of ebselen on the death of NSC34 cells on serum withdrawal. Data shown are from four repeats in three wells (WT, G93A and G37R cells) and three repeats in three wells in NTG cells and for the 20 μM dose in all four cell lines. Results shown are means ± 1 SEM. Serum withdrawal resulted in cell death in all NSC34 cell lines investigated but was most marked in G93A and G37R cells (NTG cell survival 48 h post-serum withdrawal as a percentage of control = 63.31% ± 6.97, WT cells = 57.03% ± 5.57, G93A cells = 32.85% ± 2.28, G37R cells = 39.13% ± 3.90). Ebselen at 1, 5, 10 and 20 μM was applied at the time of serum withdrawal to (A) NTG cells; (B) WT cells; (C) G37R cells; and (D) G93A cells, and 48 h later an MTT assay was performed. Ebselen treatment has a significant effect on survival in G37R (P = 3.1 × 10^{-4}) and G93A cells (P = 0.0011) but not on NTG (P = 0.19) or WT cells (P = 0.4, ANOVA).

At doses of 5 μM (P = 0.00033), 10 μM (P = 4.3 × 10^{-4}) and 20 μM (P = 5.6 × 10^{-3}), the rescue of G37R cells by ebselen is statistically significant [Student’s t-test comparing serum withdrawal (SF) with and without each dose of ebselen]. Rescue of G93A cells by ebselen is statistically significant at 5 μM (P = 0.00053), 10 μM (P = 0.0018) but not at 20 μM (P = 0.83). Rescue of G37R cells is greatest at 67.21% ± 4.2 of control, with maximal rescue provided by 10 μM ebselen. Rescue of G93A cells is 44.34% ± 1.53 of control with maximal rescue provided by 5 μM ebselen. This represents a maximal increased survival of 71.8% for G37R cells (10 μM ebselen) and of 35% (5 μM ebselen) for G93A cells. **Significant to P < 0.01, *Significant to P < 0.05. NTG = non-transgenic NSC34 cells; WT = NSC34 cells expressing wild-type human SOD1; G93A = NSC34 cells expressing G93A mutant human SOD1; G37R = NSC34 cells expressing G37R mutant human SOD1. FBS = fetal bovine serum, SF = serum-free.

mitochondria from G93A transgenic mice, however. As Prx2 is expressed in larger neurons (Kato et al., 2004) but not glia (Sarafian et al., 1999), its upregulation within motor neurons might be masked by an increased contribution to the sample by glial mitochondria in the G93A mice. Given reductions in other mitochondrial Prxs in the presence of mutant SOD1, Prx2 upregulation may represent a compensatory attempt to deal with excess H₂O₂ leaking from mitochondria lacking adequate peroxidase activity. This theory is supported by recent findings of increased Prx2 expression in residual motor neurons in spinal cord from SOD1 transgenic rats and mice and more importantly in sMND and SOD1-related fMND patients (Kato et al., 2005). The finding of Prx2 in Lewy body-like hyaline inclusions in SOD1-related fMND patients (Kato et al., 2004; Kato et al., 2005), however, raises two further interesting possibilities. First, increased Prx2 expression may represent failure to remove Prx2 sequestered in protein aggregates. Secondly, a more active role in the handling of misfolded proteins within these aggregates may be a possibility, given recent reports of chaperone function of multimers of oxidized 2-cys Prxs (Jang et al., 2004; Kang et al., 2005; Moon et al., 2005).
neurofilament in spinal cord from patients with sMND and SOD1-related fMND (Kato et al., 2001). In the second, Gal 1 was shown to be involved in the assembly of the HeLa spliceosome and was associated with Gemin4, a member of a multimeric protein complex along with the survival motor neuron (SMN) protein (Park et al., 2001). Finally, injection of recombinant, oxidized Gal 1 into the left gastrocnemius of H46R SOD1 mice produced a small but statistically significant delay in symptom onset and a modest survival benefit (Chang-Hong et al., 2005). Although the role Gal 1 plays in MND remains unclear, these studies suggest that further investigation may be warranted.

Decreased Prx3 expression may have consequences beyond a reduction in mitochondrial matrix peroxidase capability. In cultured cells stably transfected with antisense Prx3, mitochondrial membrane potential decreased and abnormalities of mitochondrial morphology developed (Wonsey et al., 2002). Perhaps reduced Prx3 contributes to NSC34 cell mitochondrial morphological abnormalities (Menzies et al., 2002) and those of G93A mice (Kong and Xu, 1998). Prx3 knockdown by siRNA also increased mitochondrial H₂O₂; reduced mitochondrial membrane potential; increased protein carbonylation; increased cytochrome c release and subsequent caspase activation and sensitized cells to staurosporine and TNF-α-induced apoptosis (Chang et al., 2004).

Western blotting of mitochondrially enriched preparations of 90 day G93A mouse spinal cord confirmed reduced Prx3 expression. No change in Prx3 expression could be demonstrated in younger mice, however. This may be due to an inability to detect subtle early changes in Prx3 within spinal motor neurons by Western blotting of mitochondria from whole spinal cord, or may instead indicate that in the presence of mutant SOD1 compensatory mechanisms able to maintain Prx3 expression for a time eventually fail and oxidative stress leads to a fall in Prx3. These experiments cannot ascertain whether Prx3 expression is reduced within motor neurons, or even whether it is present in these cells. Immunohistochemistry performed on spinal cord from 90 day G93A mice and humans, however, confirmed the presence of Prx3 in anterior horn cells and suggested that degenerating motor neurons may contain less Prx3 (Fig. 6F). We further investigated Prx3 expression in patients by performing Q-PCR on ~1000 pooled spinal motor neurons per individual. These data confirm that a decrease in Prx3...
It had been suggested that aberrant copper chemistry might underlie the oxidative stress seen in SOD1-related fMND. Ablation of the SOD1 copper chaperone (required for 90% of SOD1 copper loading), however, did not affect disease progression in SOD1 mice (Subramaniam et al., 2002). Furthermore, SOD1 mice carrying additional mutations abolishing copper-binding within the enzyme’s active site, nevertheless, developed an MND similar to that of unmodified SOD1 transgenic mice (Wang et al., 2003). This casts doubt on the hypothesis that oxidative stress caused by aberrant copper chemistry is a major mechanism in SOD1-related fMND. The findings of the present report and our recent gene expression study (Kirby et al., 2005) provide new insights into how mutant SOD1 may cause oxidative stress extending beyond its own catalytic activity, by altering the expression of key proteins involved in mitochondrial antioxidative defence and the Nrf-2-mediated cellular antioxidant response.

Ebselen (C$_{13}$H$_9$NOSe) is an anti-oxidant that acts as a Prx-mimic (Kang et al., 1998; Zhao and Holmgren, 2002). Ebselen has mitochondrially mediated anti-apoptotic effects (Narayanaswami and Sies, 1990; Boireau et al., 1999; Boireau et al., 2000) and a modulatory effect upon the mitochondrial permeability transition (MPT). In isolated rat liver mitochondria, low doses promote MPT, but higher doses inhibit MPT (Morin et al., 2003). Ebselen may also induce apoptosis (Yang et al., 2000), underscoring this double-edged sword effect. Our results and those of Tamasi et al. (2004) indicate that ebselen is able to induce the ARE in addition to its ability to reduce H$_2$O$_2$ directly via its intrinsic peroxidase activity.

We treated NSC34 cells subjected to oxidative stress by serum withdrawal with ebselen to investigate its potential neuroprotective effects. Ebselen selectively rescued G93A and G37R cells but had no such effect on NTG or WT cells. As Prx3 and Prx4 expression is reduced in both mutants, it is tempting to postulate that ebselen increases the thioredoxin-peroxidase capability of the cell above and beyond the compensatory upregulation of Prx2, to beneficial effect. In NSC34 cells stably transfected with G93A mutant SOD1, we previously found that Nrf-2 mRNA levels were 3-fold lower than in control cells (Kirby et al., 2005). As well as directly detoxifying excess H$_2$O$_2$ resulting from reductions in Prx3 and 4, ebselen may also increase the expression of anti-oxidant proteins including some Prxs and the proteins that serve to keep them in their reduced and active state via its effects on the ARE.

Modulation of mitochondrial bioenergetics is considered to offer therapeutic possibilities in MND, but, to date, results in patients have been disappointing (Mazzini et al., 2001; Shefner et al., 2004). This may be due to failure of the drugs to reach CNS mitochondria, but more likely reflects our poor understanding of the molecular basis of mitochondrial dysfunction in MND. The fact that creatine and coenzyme Q$_{10}$ are not aimed at any MND-specific target reflects this difficulty. We have demonstrated a specific deficit in mitochondrial anti-oxidant defence in two complementary MND models and in patients with sporadic and SOD1-related familial disease. Upregulation of Prx-like activity with ebselen in our cell-culture model mitigated toxicity caused by mutant SOD1. Ebselen is neuroprotective (Hattori et al., 2003), anti-oxidant (Satoh et al., 2004) and anti-apoptotic and, importantly, has been safely given to patients (Yamaguchi et al., 1998). We conclude that further consideration is warranted of the therapeutic modulation of mitochondrial anti-oxidant defence, and in particular of the use of ebselen as a neuroprotectant in MND.

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References


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