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Review

Oxidative stress in ALS: A mechanism of neurodegeneration and a therapeutic target

Siân C. Barber, Richard J. Mead, Pamela J. Shaw *

Academic Neurology Unit, Section of Neuroscience, E Floor, Medical School, Beech Hill Road, Sheffield S10 2RX, UK

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Abstract

The cause(s) of amyotrophic lateral sclerosis (ALS) is not fully understood in the vast majority of cases and the mechanisms involved in motor neuron degeneration are multi-factorial and complex. There is substantial evidence to support the hypothesis that oxidative stress is one mechanism by which motor neuron death occurs. This theory becomes more persuasive with the discovery that mutation of the anti-oxidant enzyme, superoxide dismutase 1 (SOD1), causes disease in a significant minority of cases. However, the precise mechanism(s) by which mutant SOD1 leads to motor neuron degeneration have not been defined with certainty, and trials of anti-oxidant therapies have been disappointing. Here, we review the evidence implicating oxidative stress in ALS pathogenesis, discuss how oxidative stress may affect and be affected by other proposed mechanisms of neurodegeneration, and review the trials of various anti-oxidants as potential therapies for ALS.

Keywords: Amyotrophic lateral sclerosis; Oxidative stress; Superoxide dismutase; Anti-oxidant; Clinical trials; Therapy

1. Introduction

Amyotrophic lateral sclerosis (ALS), or motor neuron disease (MND) as it is often called, is one of the more prevalent adult-onset neurodegenerative diseases, with an incidence of 1–2/100,000 in most populations. The disease is characterised by progressive injury and death of lower motor neurons in the spinal cord and brainstem, and upper motor neurons in the motor cortex. Disease progression is usually rapid, and the average survival is only 2–3 years from symptom onset. Despite extensive research, the cause of disease is unknown in the majority of cases, and the mechanisms of motor neuron injury are complex and incompletely understood. The discovery that approximately 20% of familial MND cases (5–10% of all cases are familial) are caused by mutations in copper/zinc superoxide dismutase (Cu/Zn SOD, SOD1) [1] has enabled development of animal and cell culture models, from which much of our understanding of the mechanisms of neurodegeneration in ALS has come. This work has highlighted several major mechanisms, which are not mutually exclusive. These include: oxidative stress; excitotoxicity caused by aberrant glutamate signalling; mitochondrial dysfunction; disruption of the neurofilament network and intracellular trafficking along neurofilaments; aggregation of proteins; and involvement of non-neuronal cells in the vicinity of motor neurons. One or more of these mechanisms culminate in the death of motor neurons by what is now thought to include a caspase-dependent programmed cell death pathway resembling apoptosis [2]. Here, we review the evidence for oxidative stress as a mechanism of neurodegeneration in ALS, describe how it may interact with other proposed mechanisms of neurodegeneration, and consider whether anti-oxidants should be pursued as a promising group of neuroprotective compounds.

2. Sources of oxidative stress

Reactive oxygen species (ROS) arise as by-products of aerobic metabolism [3–5]. For a comprehensive review of reactive oxygen species, we refer the reader to Halliwell and Gutteridge [5]. Most cellular ROS arise due to “leakage” of electrons from the mitochondrial respiratory chain resulting in

* Corresponding author. Tel.: +44 0114 2713579; fax: +44 0114 2261201.
E-mail address: Pamela.Shaw@sheffield.ac.uk (P.J. Shaw).

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incomplete reduction of molecular oxygen during oxidative phosphorylation to produce hydrogen peroxide (H_2O_2) and the superoxide radical anion ($O_2^{-\bullet}$, Fig. 1). Whilst cytochrome C (the terminal oxidase of the electron transport chain, and the only one that uses molecular oxygen) has a high affinity for oxygen and there is no evidence that it releases oxygen radicals [5], earlier components of the electron transport chain (notably complexes I and III) do leak some electrons to molecular oxygen [6]. It has been estimated that at physiological levels of O_2, 1–3% of molecular oxygen reduced in mitochondria may form superoxide [5]. Since approximately 85–90% of oxygen is used by mitochondria, this makes the mitochondrion the major site of ROS production. The remaining 10–15% of oxygen is used by other cellular oxidative enzymes, including xanthine oxidase in the cytoplasm and the oxidase activity of the cytochrome P450 system in the endoplasmic reticulum, which can also yield ROS (Figs. 1 and 2) [5].

Whilst superoxide itself is not highly reactive [7], it will react quickly with the nitric oxide radical ($NO^{\cdot}$), produced by nitric oxide synthase, to form the potent oxidant, peroxynitrite ($ONOO^-$, Fig. 1) [8,9]. Similarly, hydrogen peroxide is a weak oxidising agent and is generally poorly reactive, but slowly decomposes to form the highly reactive hydroxyl radical, $^{\cdot}OH$ [5]. This can be accelerated in the presence of reduced metal ions, such as ferrous ion, Fe^{2+} (Fenton reaction, Fig. 1). Both peroxynitrite and hydroxyl radicals are highly reactive and can cause oxidative damage to proteins, lipids and DNA. Such damage can alter protein conformations and disrupt enzyme active sites, change the properties of cellular membranes by oxidation of unsaturated fatty acids, and introduce mutations into DNA (Fig. 2). Some components of the electron transport chain are encoded by mitochondrial DNA, which has a 10-fold higher mutation rate than nuclear DNA [10]. The mutation rate of mitochondrial DNA increases further with age, suggesting that the oxidative environment in which it resides contributes to this increased mutation rate, and this vicious cycle of ROS production and decreased mitochondrial efficiency is considered to be a major cause of aging [11–13].

3. Anti-oxidant defence mechanisms

The cell combats this continuous production of harmful ROS with a multi-faceted anti-oxidant defence mechanism. These

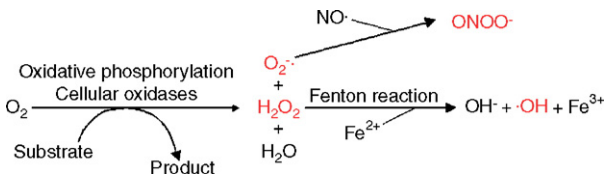

Fig. 1. Production of reactive oxygen species. Incomplete reduction of molecular oxygen during oxidative phosphorylation and other oxidative reactions leads to production of superoxide radicals ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2). Reaction between superoxide and nitric oxide (NO) produces peroxynitrite ($ONOO^-$). Hydrogen peroxide is converted to hydroxyl radical ($^{\cdot}OH$) by cytosolic transition metal cations in the Fenton reaction. ROS are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

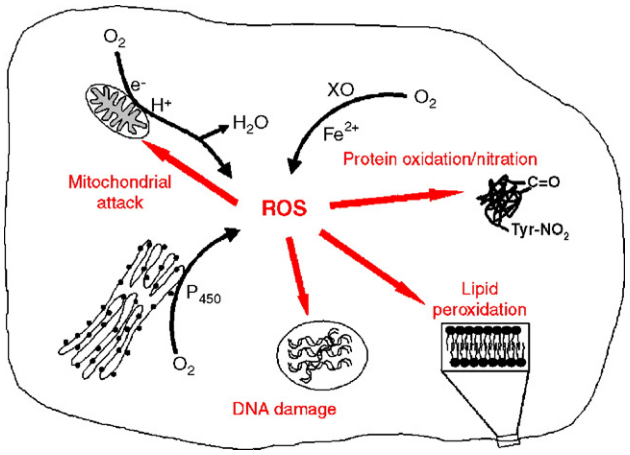

Fig. 2. Sources of reactive oxygen species and their targets. ROS are produced during oxidative phosphorylation in mitochondria, by oxidative enzymes including cytochrome P450 in the endoplasmic reticulum, and by xanthine oxidase (XO) and reduced metal ions in the cytosol. Cellular targets attacked by ROS include DNA, proteins, membrane lipids, and mitochondria.

defences include: (1) catalytic removal of reactive species by enzymes such as superoxide dismutase, catalase, and peroxidase; (2) scavenging of reactive species by low molecular weight agents either synthesised in vivo (including glutathione, α-keto acids, lipoic acid and coenzyme Q) or obtained from the diet (including ascorbate (vitamin C) and α-tocopherol (vitamin E)); (3) minimising the availability of pro-oxidants such as transition metals; and (4) protection of bio-molecules by heat-shock proteins which act to either remove or facilitate repair of damaged proteins [5]. Superoxide dismutase (SOD) enzymes catalyse the removal of superoxide radicals in a dismutation reaction where one superoxide radical is oxidised and another is reduced (Fig. 3A) [14]. Two unrelated SOD enzymes are present within eukaryotic cells; copper/zinc SOD (Cu/Zn SOD, SOD1) in the cytosol and mitochondrial intermembrane space, and manganese SOD (Mn SOD, SOD2) in the mitochondrial matrix [14,15]. Many eukaryotes also have an extracellular Cu/Zn SOD [16]. That unrelated SOD enzymes have evolved highlights the importance of anti-oxidant defence. The hydrogen peroxide that is produced can be degraded to water and oxygen by catalase (Fig. 3B.I), or can be metabolised by peroxidase enzymes, such as the glutathione peroxidases, which use reduced glutathione as a co-factor that is oxidised by hydrogen peroxide (Fig. 3B.II) [17]. The oxidised glutathione is recycled to its reduced form by glutathione reductase enzymes, thereby maintaining a high ratio of reduced/oxidised glutathione. Ascorbate acts as an anti-oxidant by donating one electron to a reactive radical to produce the much less reactive ascorbyl radical (Fig. 3C). Ascorbyl is poorly reactive, but undergoes a dismutation reaction where one ascorbyl radical donates an electron to another, with the result of regenerating one ascorbate and one molecule of dehydroascorbate, which is rapidly degraded [18]. Alpha-tocopherols react with lipid peroxyl radicals more rapidly than these radicals can react with adjacent fatty acids or membrane proteins, and therefore terminate the chain reaction of lipid peroxidation (Fig. 3D). The α-tocopherol radical produced can be recycled by reaction with ascorbate, or

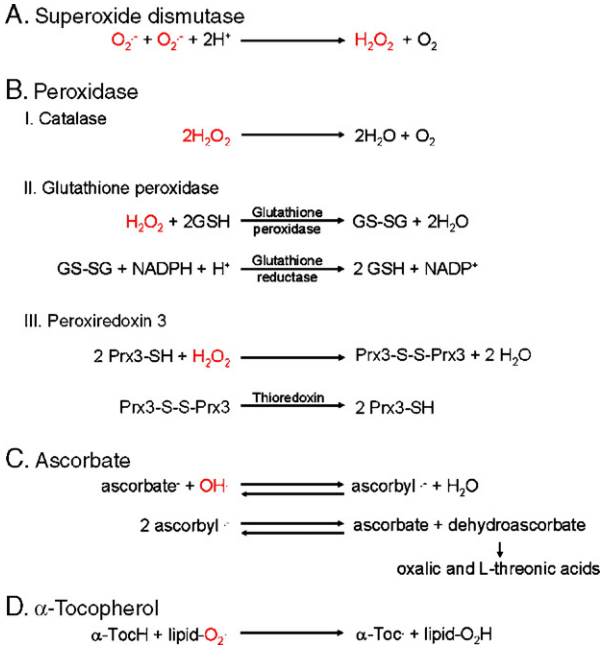

Fig. 3. Anti-oxidant mechanisms. (A) Superoxide dismutase oxidises one superoxide radical and reduces another, to produce hydrogen peroxide and oxygen. (B) Hydrogen peroxide can be degraded by catalase, glutathione peroxidases and, in mitochondria, by peroxiredoxin 3. Glutathione and peroxiredoxin 3 are recycled by glutathione reductase and thioredoxin, respectively. (C, D) Low molecular weight agents such as ascorbate and α-tocopherol can react with and remove various ROS. Ascorbyl radical is poorly reactive and is degraded to produce oxalic and L-threonic acids, as well as regenerating some ascorbate [5]. Alpha-tocopherol radical can be recycled by reaction with ascorbate, or can be further metabolised and excreted.

can be further metabolised and excreted in the urine. Proteins such as transferrin and albumin in extracellular fluids sequester transition metal ions thereby preventing them from catalysing generation of hydroxyl radicals [19].

3.1. Mitochondrial anti-oxidant defence mechanisms

As the major site of ROS generation, it is not surprising that the mitochondrion has its own anti-oxidant defence mechanisms. Superoxide dismutase again plays a major role in anti-oxidant defence, but although SOD1 is found in the intermembrane space [15,20,21] where it can remove superoxide generated at the outer face of the inner mitochondrial membrane [22], it is SOD2, present in the mitochondrial matrix, that is the major mitochondrial SOD enzyme [23,24]. Since superoxide does not readily cross cell membranes, SOD2 has a vital role in anti-oxidant defence in mitochondria, evident in that SOD2 knock-out mice develop mitochondrial deficiencies associated with ROS toxicity and die in early postnatal life [25,26]. Catalase is not present within mitochondria, so the hydrogen peroxide produced by SOD2 is degraded by glutathione peroxidases [27,28] or peroxiredoxin 3 (Prx3) [29,30] within the mitochondrial matrix. Prx3 homodimers catalyse removal of hydrogen peroxide in a mechanism reminiscent of glutathione peroxidase; hydrogen peroxide oxidises the thiol group on an N-terminal conserved cysteine residue in one Prx3 subunit, which in turn reacts with a second conserved thiol group at the C-terminal of the other Prx subunit (Fig. 3B.III). The oxidised Prx3 is then recycled by reaction with thioredoxin (Trx), which is itself recycled to its reduced form by thioredoxin reductase. Whilst the peroxidase activity of Prx3 is low compared to that of catalase and the glutathione peroxidases [29], it is more abundant within mitochondria [31], and is up-regulated in response to oxidative stress [32], making it an important factor in mitochondrial anti-oxidant defence.

4. Evidence for oxidative damage in ALS

Numerous studies have found evidence of increased oxidative stress in ALS pathogenesis. Extensive evidence shows increased oxidative damage to protein in ALS post mortem tissue compared to control samples. Protein carbonyl levels have been found to be elevated in both spinal cord [33] and motor cortex [34] from sporadic ALS cases, and increased 3-nitrotyrosine levels, a marker for oxidative damage mediated by peroxynitrite, was observed in both sporadic and SOD1 familial ALS patients [35–37]. Immunoreactivity to 3-nitrotyrosine was most prominent within large ventral horn neurons. Markers for protein and lipid oxidation were localised in motor neurons, reactive astrocytes and microglia/macrophages in the grey matter neuropil of sporadic ALS patients that were absent in control spinal cords [38]. Oxidative damage to DNA, measured by levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG), has also been found to be elevated in whole cervical spinal cord from ALS patients [39] and to be most prominent within the ventral horn [34]. Increased levels of 8-OHdG [40,41], 4-hydroxynonenal (indicative of lipid peroxidation) [42,43], and ascorbate free radical [41] have been reported in cerebrospinal fluid (CSF) samples from ALS patients. Elevated 3-nitrotyrosine levels [44] and nitrated manganese superoxide dismutase [45] have also been measured in CSF samples from ALS patients, although a more recent study found 3-nitrotyrosine was elevated in CSF from only a minority of ALS patients, and was not significantly different to control individuals [46].

Whether oxidative stress is a primary cause of pathogenesis in ALS, or is merely a consequence of the disease has long been debated. The discovery that approximately one fifth of familial ALS cases showed genetic linkage to the SOD1 locus [1] was therefore exciting, given the major role that SOD1 has in anti-oxidant defence. This finding placed oxidative stress as a central mechanism in familial ALS pathogenesis. Transgenic mouse models of ALS expressing mutant human SOD1 support the human studies showing increased oxidative damage to protein, lipid and DNA [47–52]. Indeed, one of the most severely oxidised proteins in the G93A-SOD1 transgenic mouse was found to be mutant SOD1 itself [48]. It was initially suggested that mutations in SOD1 may be toxic through either: (1) loss of function leading to increased levels of superoxide, which can react with nitric oxide to produce peroxynitrite [53,54]; (2) a dominant-negative mechanism whereby the mutant SOD1 protein is not only inactive, but also inhibits the function of normal SOD1 expressed by the normal allele [1]; or 3) increased

SOD1 activity leading to increased hydrogen peroxide levels and hydroxyl radical [1]. However, subsequent investigation found that the mechanism through which mutant SOD1 causes disease is more elusive.

5. SOD1 chemistry

Cu/Zn superoxide dismutase (SOD1, EC 1.15.1.1) is a small, ubiquitous, homodimeric protein found in the cytosol that forms a major component of the anti-oxidant response. SOD1 uses the copper ion in its active site to catalyse a two-step redox reaction that dismutates superoxide radicals to oxygen and hydrogen peroxide (Fig. 4A) [14]. Access to the active site, where the copper ion resides along with a zinc ion, is restricted. Superoxide anions are guided through a narrow channel to the small active site by highly conserved positively charged residues lining the channel [55]. This conformation encourages superoxide entry, but excludes molecules of larger size, thereby protecting the metal cations from participating in undesirable side reactions. Within the active site, the dismutation reaction occurs rapidly [56]. The uncharged hydrogen peroxide is free to leave the active site, and is subsequently converted to oxygen and water by catalase or glutathione peroxidase.

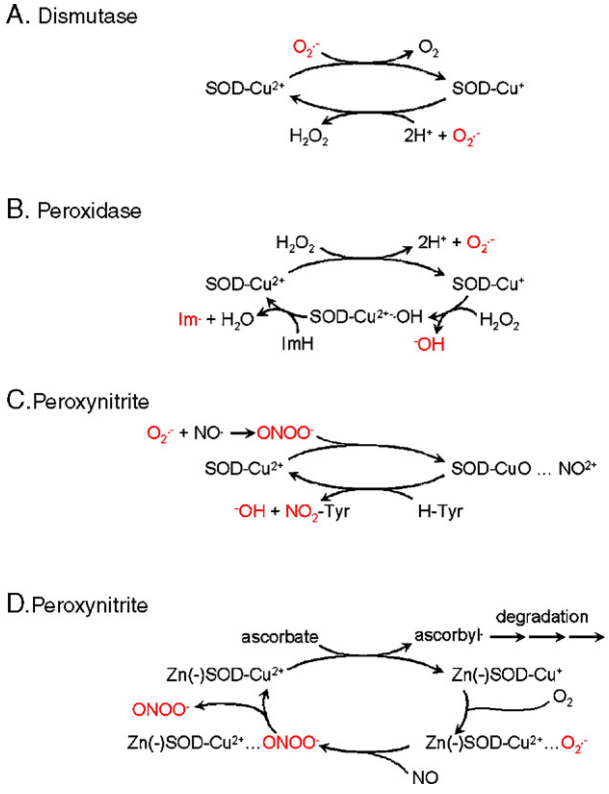

Fig. 4. SOD1 chemistry. (A) Superoxide dismutation reaction. (B) Peroxidase activity of SOD1, producing superoxide radical, hydroxyl radical, and (if performed in isolation) can irreversibly inactivate SOD1. Im, imidazole ring. (C) Elevated superoxide levels, caused by reduced SOD1 activity, lead to increased peroxynitrite production, that reacts with SOD1 leading to tyrosine nitration. (D) Zinc-deficient SOD1 is reduced by cellular reducing agents such as ascorbate, so it can then catalyse the reverse of the dismutase reaction to produce superoxide, which in turn reacts with nitric oxide to produce peroxynitrite. Cellular reductants ensure recycling of SOD1, enabling further superoxide generation.

How over one hundred different mutations in the 153 amino acid SOD1 sequence can lead to selective motor neuron death remains controversial. Initial hypotheses suggested that ALS-causing mutations in SOD1 altered the protein conformation to give a mis-folded or unstable enzyme with reduced dismutase activity thereby leading to increased oxidative stress [53]. Early experimental evidence supported this hypothesis with two independent studies reporting SOD1 activity in red blood cell lysates from fALS patients to be less than half the activity from controls [54,57], and a further report describing reduced SOD1 activity in fALS cortical tissue [58]. However, it soon became apparent that a simple loss of function could not explain mutant SOD1 toxicity, since the level of dismutase activity was found to vary between different fALS-linked SOD1 mutations [59], with some, such as the G37R mutant, retaining full dismutase activity [60]. No fALS cases have been found with mutations that lead to a total loss of SOD1. Data from SOD1 transgenic mouse models also invalidate the loss of dismutase function hypothesis: (1) SOD1 mutations that do not decrease dismutase activity are sufficient to cause motor neuron degeneration [61–64]; (2) increasing or decreasing levels of wild-type SOD1 had no effect on mutant SOD1-related ALS [65]; and (3) a SOD1 knock-out mouse did not develop ALS [66]. If mutant SOD1 does not cause fALS through loss of dismutase activity, it appears that mutant SOD1 gains an additional toxic function.

Since many of the mutations identified in SOD1 alter residues important in β-barrel formation and dimer contact rather than at the active site [54], it has been proposed that ALS-causing mutations in SOD1 may lead to a more open conformation, allowing aberrant substrates to enter the active site and react with the copper and/or zinc ions sequestered within. Two hypotheses have been proposed for this new toxic function. Mutant SOD1 could act as a peroxidase in the reverse of its normal reaction, or could react with peroxynitrite to cause tyrosine nitration (Fig. 4B–D).

In addition to its dismutase activity, SOD1 can also act as a peroxidase by using the hydrogen peroxide produced in the conventional dismutase reaction as a substrate. In this way, SOD1 can catalyse the reverse reaction of the dismutation, and the reduced copper ion can then use more hydrogen peroxide to produce hydroxyl radicals through the Fenton reaction (Fig. 4B) [67–69]. When this reaction occurs in isolation, the SOD1 rapidly becomes inactivated because the bound hydroxyl radical attacks and oxidises the imidazole ring on the adjacent histidine residue to which the copper ion is bound [67]. However, other small electron donors, such as formate, glutamate and urate, are able to pass through the positively charged channel to the active site where they can compete with the imidazole ring to scavenge the highly reactive hydroxyl radicals, so that the peroxidase activity of SOD1 can continue [67,69]. Such free-radical generation by SOD1 has been implicated in ALS; the G93A-SOD1 mutation retains dismutase activity equivalent to wild-type SOD1 [61], but has a greater free radical generating activity [70].

Mutations in SOD1 may result in increased peroxynitrite levels through two hypothesised mechanisms. The first hypothesis assumes that the mutant SOD1 activity is lower

than normal SOD1 activity, causing a shift in the steady-state equilibrium towards higher superoxide concentrations. Since superoxide reacts with nitric oxide more rapidly than it does with native SOD1, there would be a concomitant increase in peroxynitrite levels, which in turn reacts with SOD1 to form a nitronium-like intermediate that nitrates tyrosine residues on cellular proteins (Fig. 4C) [53]. Although increased nitro-tyrosine levels have been reported in SOD1-ALS patients [37,44] and mutant SOD1 transgenic mice [47,52], it is now understood that many SOD1 mutants retain dismutase activity [59,60,71] so this mechanism could only occur in mutants where dismutase activity is reduced.

The second hypothesis is based on the finding that several ALS-linked SOD1 mutations, including A4V and I113T, show a reduced affinity for zinc [72,73]. When replete with both copper and zinc, neither wild-type nor four ALS-SOD1 mutants were found to be toxic to cultured motor neurons, but when they were made zinc-deficient (Zn(−)SOD1), all induced apoptosis in motor neuron cultures [74]. Removal of copper protected neurons from Zn(−)SOD1, suggesting the normal dismutase activity of SOD1 contributes to toxicity. The altered geometry associated with Zn(−)SOD1 enabled reductants other than superoxide, including ascorbate, glutathione and urate, to enter the active site and react rapidly with oxidised Cu^{2+} [74]. The now-reduced Cu^{+} is able to catalyse the reverse of its usual dismutase reaction, using oxygen to generate superoxide, which in turn reacts with freely diffusing nitric oxide to produce peroxynitrite and cause tyrosine nitration as described previously [53] (Fig. 4D). Therefore, Zn(−)SOD can continue to produce superoxide at the expense of cellular anti-oxidants [74]. If the superoxide produced in this way is not released from Zn(−)SOD1, but reacts with nitric oxide to produce peroxynitrite, it would explain why an excess of wild-type SOD1 cannot compensate for this aberrant redox activity [65]. It is interesting that A4V, a mutant associated with rapid disease progression [75], showed the lowest zinc affinity of mutants studied [73].

The importance of peroxynitrite synthesis in mutant SOD1 toxicity remains controversial. If such a mechanism was central to the toxic gain of function, it would be hypothesised that reduction of nitric oxide levels by inhibition of neuronal NO synthase would reduce mutant SOD1 toxicity to motor neurons. Although this was found in one study using cultured motor neurons [74], inhibition of neuronal NO synthase had no effect on survival of mutant SOD1 transgenic mice [76,77]. Similarly, if increased peroxynitrite was a major mechanism leading to neurodegeneration, increased levels of tyrosine nitration would be expected. Elevated levels of free 3-nitrotyrosine have been found in G37R- and G93A-SOD1 transgenic mice compared to mice expressing human wild-type SOD1 [47,78], and also in both sporadic and SOD1-ALS patients [37]. However, levels of protein-bound nitro-tyrosine were unchanged at all ages in G37R- and G85R-SOD1 mice, and also in end-stage of sporadic or mutant SOD1-mediated human ALS [78].

All of the proposed mechanisms for the mutant SOD1 gain of toxic function require a copper ion to be present within the active site. In support of this, neither loss of dismutase activity nor reduced copper binding is a common property shared by ALS-associated SOD1 mutants [59,71]. However, deletion of the copper chaperone for SOD1 (CCS), which led to a reduction in copper loading into SOD1 of at least 90% and a marked reduction of SOD1 activity, had no effect on disease onset, progression or pathology of G93A, G37R or G85R mutant mice [79]. Similarly, mutation of the four histidine residues that hold the copper ion in the active site (including the two disease-causing mutations, H46R and H48Q) abolished the copper binding site and produced a completely inactive protein, but still caused a motor neuron disease in a transgenic mouse that was clinically and pathologically similar to other mouse models of ALS [80]. These studies showing that enzymatically inactive SOD1 is capable of causing motor neuron degeneration cast doubt on the hypothesis that oxidative stress caused by aberrant copper chemistry is a common mechanism in ALS pathology. Since a SOD1 knock-out mouse does not develop motor neuron disease [66], and oxidative damage is a well characterised feature of ALS pathology (see above), it seems plausible that mutant SOD1 may induce oxidative stress in a mechanism beyond its own catalytic activity. In support of this, recent studies showed that expression of mutant SOD1 in a motor neuronal cell line altered the gene expression profile and caused a decrease in the cellular anti-oxidant response [81,82]. In the presence of mutant SOD1, NSC34 motor neuronal cells underwent transcriptional repression, with genes involved in anti-oxidant responses representing one of the major groups of genes to show decreased expression [82]. Several members of the glutathione S-transferase family, two peroxiredoxins, glucose-6-phosphate dehydrogenase, an aldo-keto reductase, and a leukotriene dehydrogenase all showed decreased expression in the presence of mutant SOD1. The expression of many of these “programmed cell life” genes involved in the anti-oxidant response is regulated by the transcription factor NRF2 [83], which acts through the anti-oxidant response element (ARE) [84,85]. In the presence of mutant SOD1, the expression of the transcription factor NRF2 was reduced 3-fold [82], suggesting that increasing NRF2 signalling may help to reverse many of these changes. In support of this, pharmacological activation of the ARE to increase expression of these genes partly protected against cell death following oxidative stress induced by serum withdrawal in a cellular model of SOD1-related ALS [82].

6. Cross-talk with other mechanisms of neurodegeneration

Oxidative stress is just one of several proposed mechanisms implicated in motor neuron injury in ALS. Other mechanisms that may contribute to pathogenesis include excitotoxicity, mitochondrial dysfunction, protein aggregation, cytoskeletal dysfunction, and involvement of non-neuronal cells, which are discussed in more detail in other reviews within this issue. These mechanisms are not mutually exclusive and it seems probable that they all participate to some extent in disease progression. Given the large number of genes implicated in familial ALS [86,87] and the lack of a specific cause of sporadic

ALS, it seems likely that a small insult tips the balance away from equilibrium and starts a vicious cycle leading to motor neuron degeneration. How oxidative stress could be caused by, and exacerbate other proposed mechanisms is discussed below (Fig. 5).

6.1. Oxidative stress and excitotoxicity

Excitotoxicity occurs when excessive stimulation of glutamate receptors, the major excitatory neurotransmitter in the mammalian central nervous system, triggers increased calcium signalling leading to neuronal injury and death [88,89]. Motor neurons appear to be particularly vulnerable to excitotoxicity mediated by AMPA receptors. There is evidence for increased glutamate signalling in a sub-set of ALS patients [90–93] and in mutant SOD1-expressing rodent models [64,94,95]. Similarly, elevated calcium levels have been found in motor nerve terminals in ALS patients [96] and mutant SOD1 mice [97]. Generation of reactive oxygen species is instrumental in glutamate-mediated toxicity. Calcium up-take into mitochondria triggered production of ROS [98], and glutamate-induced degeneration of forebrain neurons was found to be triggered by up-take of calcium into mitochondria, rather than by the elevated levels of intracellular free calcium [99]. Similarly, calcium loading into motor neuron mitochondria, by stimulation with AMPA, was found to trigger mitochondrial depolarisation and ROS generation [100].

Reactive oxygen species may in turn be able to trigger excitotoxicity. Glutamate uptake through both glial and neuronal glutamate transporters was reduced by exposure to ROS [101–103]. The peroxidase activity of mutant SOD1 was

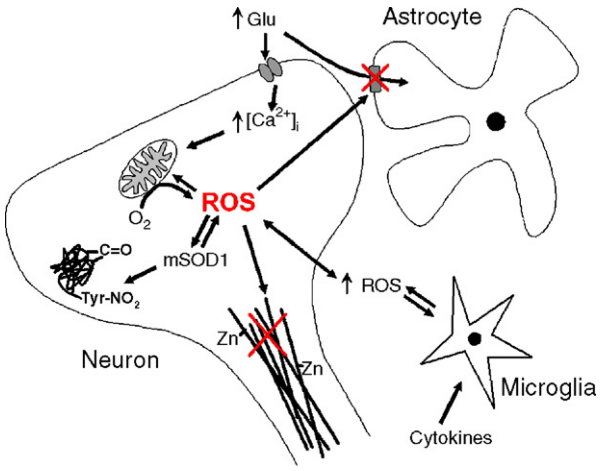

Fig. 5. Oxidative stress potentially influences other proposed mechanisms of neurodegeneration in ALS. Excitotoxicity leads to increased intracellular calcium levels that are buffered by mitochondria leading to increased ROS production. ROS, in turn, inhibit glutamate uptake through the EAAT2 transporter in glial cells. ROS can also cross the cell membrane and activate microglia, which respond by releasing cytokines and further ROS. Aberrant oxidative reactions catalysed by mSOD1 increase production of the highly reactive peroxynitrite and hydroxyl radical, causing nitration and aggregation of proteins including mSOD1 itself, and may also inhibit neurofilament assembly and cytoskeletal transport. Zinc binding to neurofilaments could deplete zinc binding to mSOD1 and exacerbate aberrant SOD1 chemistry.

sufficient to cause oxidation and inactivation of the EAAT2 glutamate transporter in *Xenopus* oocytes [104] and EAAT2 modified by the lipid peroxidation product 4-hydroxynonenal has been reported in sporadic ALS patients [105]. Inactivation of glutamate transporters could lead to persistent high levels of glutamate in the synapse, triggering excessive activation of AMPA receptors, increased intracellular calcium levels and ROS generation.

Excitotoxicity and oxidative stress could combine to form a feed-forward cycle in which glutamate stimulates increased influx of calcium into the motor neuron, which is taken up by mitochondria which in turn results in increasing ROS generation. The ROS are able to exit the cell and inactivate glutamate transporters resulting in increased glutamate levels in the synapse which in turn trigger activation of AMPA receptors and calcium influx [106]. This vicious cycle could be initiated at any point in the loop and would ultimately culminate in motor neuronal death.

6.2. Oxidative stress and mitochondrial dysfunction

There is a substantial body of data that indicate mitochondrial dysfunction is a feature of motor neuron degeneration in ALS [107]. Morphologically abnormal mitochondria have been observed in motor neurons from ALS patients [96,108,109], mutant SOD1 transgenic mice [63,110,111] and a motor neuronal cell line expressing mutant SOD1 [112]. Biochemical studies have revealed decreased electron transport chain activity [112–115], reduced mitochondrial membrane potential [116], altered calcium homeostasis [116,117], and changes to the mitochondrial proteome [118]. As the major source of reactive oxygen species (discussed earlier), mitochondria are implicated in production of oxidative stress, and are also targets of ROS, with decreased mitochondrial efficiency caused by oxidative damage being considered a major cause of aging [11–13]. Elevated levels of ROS have been proposed to cause the increased frequency of mitochondrial DNA mutations in the motor cortex and spinal cord of ALS patients [115,119], and could be responsible for the increased translocation of cytochrome *c* from mitochondria into the cytoplasm, a trigger for apoptosis, during disease progression in G93A-SOD1 mice [120,121].

Although SOD1 is generally considered to be a cytosolic protein [14], it has recently been established that there is a pool of enzymatically active SOD1 in the mitochondrial intermembrane space [15,20,21]. Mutant SOD1 has also been found within mitochondria, and such accumulation of mutant SOD1 preceded onset of symptoms in SOD1 transgenic mice [122], and coincided with increased oxidative damage, decreased respiratory activity of mitochondria [113] and the appearance of mitochondrial swelling and vacuolisation [123]. The mechanism by which SOD1 enters the mitochondrion is unknown, but it seems plausible that it is stimulated by the oxidative damage that accumulates with age [11–13]. Localisation of mutant SOD1 to mitochondria was sufficient to cause death of neuroblastoma cells [124], and high molecular weight aggregates of SOD1 that sequester the anti-apoptotic protein Bcl-2

have been observed [125], which could trigger programmed cell death [126].

6.3. Oxidative stress and protein aggregation

Mis-folded mutant proteins that form aggregates are a common feature of many neurodegenerative diseases including ALS [65,127–129], although whether they play a role in disease pathogenesis, are harmless by-products, or represent a defence mechanism by sequestration of harmful intracellular proteins is not known [130–132]. Both mutant SOD1 transgenic mice [61,64,65,110,133,134] and cell culture models [135–137] have also been found to develop protein aggregates. How SOD1 mutants with defects in the metal binding region, such as G85R, form aggregates is relatively straight forward. The structure of SOD1 is stabilized by the bound metal ions, and SOD1 mutants with reduced metal ion binding show decreased thermal stability [138] and an increased tendency to aggregate [139]. Explanation of how aggregation of "wild type-like" mutants, like A4V and G93A, occurs is more difficult, although oxidative stress has been implicated as a contributory factor. Whilst SOD1 mutants with full metal ion binding and "wild type-like" activity did not aggregate under control conditions, oxidation of these mutants led to aggregation [139]. Since aberrant oxidative reactions catalysed by metal-bound SOD1 mutants caused oxidative damage to SOD1 itself [48,67,68], it has been proposed that such oxidative damage could lead to aggregation of mutant SOD1 [140].

6.4. Oxidative stress and cytoskeletal dysfunction

Neurofilaments constitute a major component of the intermediate filament network within neuronal cells, and are responsible for maintaining cell shape and axonal calibre. Abnormal accumulation of neurofilaments is a pathological feature of many neurodegenerative disorders including ALS [141,142] and has also been seen in SOD1 transgenic mice [61,63,133,143–145]. Neurofilament subunits, classified according to their molecular weight as light (NF-L), medium (NF-M) or heavy (NF-H), have been identified as targets for oxidation by ROS produced by mutant SOD1. Disassembled NF-L subunits aggregated in response to the peroxidase activity of SOD1 [146], and axonal transport of neurofilament subunits was inhibited by the activity of the cyclin-dependent kinase Cdk5 in response to oxidative stress [147]. Tyrosine residues in disassembled NF-L subunits were selectively nitrated by SOD1, and these nitrated NF-L subunits inhibited the assembly of unmodified neurofilament subunits [148]. Since NF-L is required for assembly of NF-M and NF-H into neurofilaments [149], nitration of NF-L could seriously disrupt the maintenance of the neurofilament network. NF-L has also been shown to have a sufficiently high affinity for zinc to potentially remove zinc from both wild-type and mutant SOD1, particularly mutants with reduced zinc binding [73]. Given that zinc depleted-SOD1 is more efficient at catalysing peroxynitrite-mediated tyrosine nitration, a vicious cycle of SOD1-catalysed tyrosine nitration and zinc depletion from SOD1 by NF-L could potentially develop [73,148].

6.5. Oxidative stress and non-neuronal cells

Evidence is now emerging for involvement of non-neuronal neighbouring cells in the pathogenesis of ALS. Activated microglia and astrocytes have been observed in spinal cord from ALS patients [150] and mutant SOD1 transgenic mice [151,152], and several inflammatory mediators have been reported to be up-regulated in the spinal cord of ALS patients [153,154] and mutant SOD1 mice [155–158]. Although mutant SOD1 was toxic to cultured primary motor neurons and motor neuronal cell lines [112,135], no motor deficit or motor neuron degeneration was observed in transgenic mice with mutant SOD1 expression restricted to neuronal cells [159,160]. This suggested that non-neuronal cells play an essential role in disease pathogenesis. However, expression of mutant SOD1 restricted to astrocytes was also not sufficient to cause disease [161], indicating that both neuronal and non-neuronal cells are required for mutant SOD1-mediated neurodegeneration. In support of this, in chimeric mice, composed of both normal and mutant SOD1-expressing cells, normal motor neurons surrounded by non-neuronal cells expressing mutant SOD1 developed pathology analogous to ALS, whereas normal non-neuronal cells increased survival of mutant SOD1-expressing motor neurons [162]. Reactive oxygen species produced within the motor neuron can cross the cell membrane [5] and activate neighbouring astrocytes and microglia [163], which in turn produce pro-inflammatory cytokines and further reactive oxygen species, which again spread to neighbouring cells [164–166]. This mechanism could contribute to the process by which motor neuron degeneration starts focally but gradually spreads to contiguous groups of motor neurons [167].

7. Selective vulnerability of motor neurons

Why motor neurons are selectively vulnerable in ALS, particularly in mutant SOD1-associated disease, remains an enigma. The vast majority of neuronal cells are more susceptible to oxidative stress than other cellular populations because they are post mitotic, and damage is going to accumulate throughout life. Motor neurons in particular are highly specialised cells, and it seems likely that this specialisation renders them vulnerable to injury [168]. Motor neurons are exceptionally large cells with a cell body of approximately 50–60 μm and an axon of up to 1 m long in humans. Maintaining a cell of this size requires a huge metabolic input, necessary for the production and transport of cellular components, maintenance of a membrane potential along the length of the axon, and action potential generation. The unusually high energy demand of the motor neuron must be met by a large complement of mitochondria, with the side effect of increased reactive oxygen species generation. As the vicious circle of increased ROS production and decreased mitochondrial efficiency develops with aging [11–13], it may be that the high

energy demand of motor neurons makes them particularly vulnerable to oxidative stress. This may explain why the proportion of SOD1 that entered mitochondria was greater in brain than in liver [113]. Mitochondria in motor neurons also have a greater role in calcium buffering than in other cells, since motor neurons express lower levels of cytosolic calcium binding proteins (CaBP, such as parvalbumin and calbindin-D28k) than other neuronal populations, which act to attenuate calcium signalling [169–171]. Low expression of CaBP in motor neurons may force mitochondria to take up more calcium ions than in other cell types, triggering increased ROS production [98,100], and may help to explain why addition of glutamate to cultured motor neurons caused greater increases in ROS than in other spinal neurons [163]. In support of this, there appears to be a correlation between low CaBP expression and susceptibility to ALS [169–172].

This potentially high ROS production by motor neuron mitochondria may explain why SOD1, which is ubiquitously expressed, is particularly abundant in motor neurons and is present in axons and dendrites as well as the cell body [173]. The high levels of SOD1, combined with the probability that SOD1 has a long half-life in motor neurons (it is transported anterogradely in the slow component and must have a half-life of sufficient length to enable it to travel along the length of the axon [174]), increase the likelihood of potential ROS production by mutant SOD1 in motor neurons and concomitant oxidative damage to neurofilaments and SOD1 itself [139]. The extensive neurofilament network and large amount of plasma membrane required because of the specific morphological features of motor neurons provide targets for oxidative modification.

Oxidative stress is capable of causing considerable damage to motor neuron populations, and can also influence other mechanisms implicated in neurodegeneration in motor neuron disease. Therefore, whether oxidative stress is a primary cause of disease or a downstream consequence of an earlier toxic insult becomes almost irrelevant, since it has the ability to cause massive harm by itself. In the absence of a definitive cause of neurodegeneration in the majority of ALS cases, it seems sensible to attempt to target the causes and consequences of oxidative stress for therapeutic intervention.

8. Oxidative stress as a therapeutic target

Oxidative stress has a long history as a therapeutic target in ALS. The first ‘trial’ of anti-oxidant therapy was reported in 1940 [175]. This paper relates experience with vitamin E in combination with wheat germ oils, bile salts, vitamin B complex and vitamin E containing foods in ALS patients. This trial does not conform to the current standard of randomised, double-blind, placebo controlled trials, and few conclusions can be drawn from this descriptive study. However, some 65 years later, a Cochrane systematic review of randomised controlled trials of antioxidant therapy in ALS failed to find any beneficial effects in the studies which fulfilled the strict inclusion criteria for the meta-analysis [176]. The authors concluded that there was no significant evidence to support the benefit of anti-oxidant therapy in ALS, but that the quality and design of the clinical studies was generally poor.

When considering the testing of any therapeutic concept, a negative result does not necessarily falsify a hypothesis and it is critical to understand the pharmacokinetics and pharmacodynamics of any agent and to power studies in such a way that they are likely to detect an effect if it is there. Any in vivo trial is as much a test of the drug as the therapeutic concept. In the following section, we have not attempted to be exhaustive, but cover some examples of common therapeutic strategies which have so far been pursued for amelioration of oxidative stress in ALS, and look to possible future directions in this field. In summary, we contend that data generated so far in human trials has not sufficiently addressed whether this pathway is relevant to progression of disease in humans. There is a lack of powerful anti-oxidants able to access the CNS which are available for clinical testing. In the industrial R&D setting, there is one promising candidate in early phase clinical trials (AEOL10150) and one candidate licensed by Merck but at a very early stage of development (carboxyfullerenes). In the academic research arena, a number of potentially exciting ways forward have been identified, but these must be pursued with rigorous attention to pre-clinical and clinical trial design, pharmacology and pharmacokinetics.

9. Completed clinical trials in ALS patients

9.1. Vitamin E

Vitamin E (α-tocopherol) is the most potent naturally occurring scavenger of ROS and reactive nitrogen species (RNS) inhibiting radical chain propagation within lipid domains as it is oxidised to α-tocopheroxyl [177]. Desnuelle et al. carried out a randomised, placebo controlled double-blind study comparing vitamin E 500 mg (Toco 500) twice daily versus placebo, in combination with riluzole in 289 patients with ALS duration of less than 5 years. There was no effect on the primary outcome measure (rate of deterioration of function assessed by the modified Norris scale) and survival was not influenced over the 12-month trial period. The patients were, however, less likely to progress to a more severe state of disease as assessed by the ALS health state scale [178]. More recently, Graf et al. tested the efficacy of a ‘megadose’ of vitamin E (5000 mg daily) in a double blind, placebo-controlled, randomised, stratified parallel-group clinical trial of 18 months duration. One hundred and sixty patients receiving riluzole were randomised to either vitamin E or placebo. No effect was detected on the primary endpoint (time to death, tracheostomy or permanent assisted ventilation) although the study was powered to detect a 50% improvement which, as the authors state, may have been too ambitious. Functional tests showed a marginal trend in favour of vitamin E and no significant side effects were seen [179].

At face value, these data detract from the hypothesis that oxidative stress is an important mediator of disease progression in ALS. There is, however, a hidden assumption that this dose of vitamin E is sufficient to ameliorate oxidative stress in the

target organ. A dose response study of α-tocopherol levels in plasma and ventricular CSF of Parkinson's disease patients suggests that, despite its lipophilicity, vitamin E does not readily penetrate the CNS. Five patients with omaya catheters received oral α-tocopherol, daily over 5 months starting at a dose of 400 IU. The dose was subsequently increased every month to 800, 1600, 3200 and 4000 IU/day. Plasma and ventricular CSF levels of α-tocopherol were determined at baseline and at the end of each month. Plasma levels showed a good dose response with levels increasing from approximately 18.8 μM at baseline to 110.5 μM at 4000 IU/day. In contrast, there was no increase above baseline of ventricular CSF levels of α-tocopherol at any dose. Baseline levels were 0.11 μM and even at supraclinical doses (4000 IU/day), these levels were statistically unchanged at 0.16 μM [180]. In a recent paper examining the activity of several antioxidants in a range of assays, α-tocopherol emerged as having an IC_{50} (concentration at which a 50% inhibitory effect is seen) of 1.5 μM in a glutamate-induced oxidative neuronal cell death assay, 4.6 μM in a DPPH (2,2-diphenyl-1-picrylhydrazy) radical scavenging assay and 59 μM in a lipid peroxidation assay [181]. Based on these data, it is perhaps not surprising that even very high doses of vitamin E have failed to show any benefit, not only in ALS but also in other neurodegenerative diseases [182].

9.2. Selegiline

Selegiline hydrochloride (Eldepryl) is a monoamine oxidase-B inhibitor with antioxidant properties, which has some therapeutic benefit in Parkinson's disease. This agent has been tested over a 6-month period in a double-blind, placebo-controlled study of 133 patients with classical ALS and symptoms for less than 3 years. Using rate of change in the Appel ALS score (AALS) as the primary outcome measure, no difference was observed between the two groups with a monthly rate of change of 3.4 points for the selegiline group and 3.5 for the placebo group. Survival analysis based on worsening of 22 points in the AALS score was also non-significant [183]. It is known that at the doses used in this trial (5 mg twice daily), almost 90% of monoamine oxidase-B activity is inhibited in the human brain [184]. This would suggest that monoamine oxidase-B plays no role in the pathogenesis of ALS. Whether the antioxidant functions of selegiline are fully active at these doses within the CNS is less clear. The proposed anti-oxidant function of selegiline is related to its ability to act as an inducer of catalase and superoxide dismutase within the CNS. The data observed in rodents is conflicting, possibly attributable to age and species or strain differences and the fact that induction of these enzymes by selegiline follows a bell shaped dose–response curve. A recent study looked at the effect of selegiline dosed subcutaneously (sc) at 2 mg/kg daily on activities of catalase (CAT), SOD1 and SOD2 (Mn-SOD) in the striatum, cortex and hippocampus of 8- and 25-week-old rats. At these doses, which are higher than those used clinically, selegiline significantly increased CAT and SOD2 activities in the striatum, but not in the cortex and hippocampus, of 25-week-old rats and had no effect on any enzyme activity in 8 week old rats. All increases in expression were less than 2-fold [185]. Although one would need to examine effects on anti-oxidant systems of upper and lower motor neurons to assess the potential of this drug as an anti-oxidant in ALS, it would be difficult to argue that the clinical trial of selegiline has fully addressed this potential mechanism. Indeed, the authors of the trial conclude that two possible reasons for the lack of effect of selegiline on oxidative mechanisms may be insufficient exposure of spinal motor neurons to the drug or that its primary mechanism of action may not be on the oxidative system [183]. In Parkinson's disease the most likely mechanism of action of the drug is selective inhibition of MAO-B, resulting in enhanced levels of phenylethylamine and dopamine but not of serotonin or noradrenaline in the basal ganglia [186].

9.3. N-acetyl cysteine

N-acetyl cysteine is a precursor of glutathione, a naturally occurring intracellular anti-oxidant, which is used clinically for the treatment of paracetamol poisoning and as a mucolytic agent [187]. A double-blind, randomised, placebo controlled trial of N-acetyl cysteine has been conducted in 110 patients with ALS [188]. N-acetylcysteine was self-administered by subcutaneous infusion at a dose of 50 mg/kg (1 ml/kg) daily for 1 year and a moderate, but statistically insignificant increase was seen on the primary end-point of survival. Secondary outcome measures were designed to assess the rate of disease progression and included muscle strength testing, myometry and forced vital capacity. These were also statistically unchanged. The study was designed to detect a 50% improvement in survival ratio at 1 year with a power of 80% and, as the authors state, the power to detect smaller effects was limited. In addition when subgroup analysis was performed there appeared to be opposite effects on survival in patients with limb and bulbar onset. The authors of the study state that N-acetyl cysteine is able to reach cerebrospinal fluid concentrations of up to 3 mg/l, 2–3 h after a subcutaneous dose of 50 mg/kg although no data is presented or cited to support this. This is a very high concentration equivalent to 18.3 μM, and compares favourably with an in vitro IC_{50} of 6.5 μM in a glutamate-induced oxidative stress assay in neurones [181], although other in vitro potency data are not available as far as we are aware. Although this is a well conducted trial, the combined limitations of statistical power and the potentially opposing effects in different subtypes of MND mean that further trials would be required to definitively judge the therapeutic potential of this approach.

10. Ongoing clinical trials

10.1. Co-enzyme Q10

Co-enzyme Q10 (CoQ_{10}) is an isoprenoid lipid which is an essential cofactor for the electron transport chain within mitochondria and also functions as a lipid soluble anti-oxidant. Levels of CoQ_{10} decrease with aging [189]. Distribution studies of high specific activity radio-labelled CoQ_{10} and its metabolites in young rats demonstrated efficient uptake into leukocytes,

spleen and liver with virtually no uptake in kidney, muscle and brain. At the subcellular level, the vast majority of CoQ_{10} was found in lysosomes presumably because membranes in non-pathophysiological conditions cannot accommodate more CoQ_{10} [190]. In 12-month-old rats, however, feeding with CoQ_{10} for 2 months at 200 mg/kg/day in food restored CoQ_{10} levels to those seen in young rats with a ~30% increase in non-perfused cerebral cortex. In cerebral cortex mitochondria, there were no significant increases after 7 or 30 days feeding, but at 60 days there was a similar enhancement of CoQ_{10} content [191]. This study went on to look at the effects of dietary supplementation with CoQ_{10} on the $SOD1^{G93A}$ mouse model of ALS. In these mice, feeding with CoQ_{10} at a rate of 200 mg/kg daily in food prior to onset of symptoms (50 days of age) had a small effect on survival which was increased from 135 days in control mice to 141 days in CoQ_{10} fed mice [191].

Co-enzyme Q10 has been taken forward into small scale clinical trials in ALS patients. The data are unpublished as far as we are aware but a brief outline is reported on the ALS association website (http://www.alsa.org/patient/drug.cfm.). A pilot study of 'high dose' CoQ_{10} has been completed in 9 patients with ALS and the treatment was well tolerated with no major adverse events. A second pilot trial with magnetic resonance spectroscopy (MRS) imaging was conducted but no further information is available. Enrolment for a larger 9-month trial of 'high dose', 'very high dose' and placebo began in October 2004 and is presumably ongoing. From the animal data it appears that at best supplementation will restore CoQ_{10} levels in mitochondria to levels seen under normal conditions, which leads to a relatively mild effect in the $SOD1^{G93A}$ preclinical model of ALS. Whether this effect translates to the clinic remains to be seen.

10.2. AEOL 10150

AEOL 10150 is a new generation manganese porphyrin that can catalyse the decomposition of oxidative species by cycling between Mn (III) and Mn (IV) states. Originally developed as a SOD mimetic, it also has the capacity to scavenge peroxynitrite. It has been designed to maximise anti-oxidant properties whilst minimising possible side-effects [192]. Daily administration of this compound (2.5 mg/kg) by the intraperitoneal or subcutaneous route from symptom onset (~90 days) in the $SOD1^{G93A}$ mice led to a significant increase in survival interval, measured as time from onset to endstage, of 2.5- to 3-fold in several experiments. Co-administration of creatine and rofecoxib did not enhance survival beyond that seen in AEOL 10150 treated mice alone. The overall extension in lifespan was 26%—impressive for a pharmacological agent dosed at such a late stage. Analysis of oxidative damage by immunohistochemistry demonstrated reduced levels of both protein bound nitrotyrosine and lipid oxidation in spinal cord of drug treated mice 10 days after initiation of treatment.

Aeolus pharmaceuticals are currently developing AEOL 10150 for use in ALS and have been encouraged by the Food and Drug Administration (FDA) to apply for fast-track status which will allow them to conduct a single pivotal phase II/III trial (company press release, see http://www.aeoluspharma.com/). A Phase I escalating single dose study has already been conducted in ALS patients investigating doses up to 75 mg with no serious adverse events. A multiple dose study is underway as of September 2005 investigating doses of 40, 60 and 75 mg twice daily by the subcutaneous route for 7 days with a single dose on the final day (data presented at the Rodman and Renshaw Techvest 7th Annual Healthcare Conference, available at http://www.wsw.com/webcast/rrshq7/aols/ until February 2006). Indirect analysis of CNS penetrance of the compound will be performed by analysis of CSF. These doses are equivalent to ~2 mg/kg daily which is comparable to the doses used in the mouse study. The $SOD1^{G93A}$ transgenic mouse data with AEOL 10150 are probably the strongest pharmacological evidence for a causative role of oxidative stress in this model and proper translation of this therapeutic paradigm to humans will hopefully shed light on its relevance in ALS.

11. Pre-clinical development

11.1. Fullerenes

Fullerenes, or 'buckyballs' as they are better known, are spherical small molecules which have significant anti-oxidant capacity. They contain multiple C=C double bonds containing unpaired electrons which can mop up oxidative species as well as catalytically dismutating superoxide molecules [193]. Various derivatives have shown activity in cell culture model systems of CNS insults [194] and C3, a tris-malonic acid derivative of buckminsterfullerene (C60), has been tested in the G93A model of ALS. When delivered by intraperitoneally implanted osmotic mini-pumps from 73 days of age, C3 dosed mice had an 8-day extension in survival and scored better on measures of motor performance [195]. In October 2003 Merck and Co signed a license with a small biotechnology company called C Sixty to develop fullerene anti-oxidants in two undisclosed therapeutic areas. Whether one of these therapeutic areas is ALS is unknown at present.

11.2. NRF2

A major part of the cells response to oxidative stress is to increase expression of anti-oxidative defence systems. The cell achieves this via activation of nuclear factor related erythroid-2 like-2 protein (NRF2), a transcription factor which interacts with the anti-oxidant response element (ARE) enhancer sequence, to promote expression of proteins involved in glutathione biosynthesis, redox proteins such as thioredoxin and phase II drug metabolising enzymes [196]. NRF2 is regulated by Kelch-like ECH-associated protein 1 (Keap1) which binds to NRF2, preventing its translocation to the nucleus. Keap 1 contains a number of cysteine residues and when these are modified by oxidative stress, NRF2 may be released allowing it to enter the nucleus and activate ARE directed transcription [197]. In this way, the NRF2–Keap1

interaction serves as an intracellular sensor of oxidative stress. Other classical signal transduction pathways can also lead to activation of NRF2 including the major intracellular signalling cascades involving MAP kinases, such as ERK, PI3 kinases and protein kinase C (PKC) [196]. A number of small molecules have been found to activate this pathway and although structurally diverse they all tend to be nucleophiles which can react with sulphydryl groups. There has been interest in these molecules as chemopreventive agents in cancer for several years [198] and more recently as therapeutic agents in neurodegenerative diseases [199]. There are a number of molecules which can activate this pathway falling into two main categories; dietary flavonoids and cyclic sulphur containing compounds. The green tea catechin-(−) epigallocatechin-3-gallate (EGCG) is a flavonoid which shows neuroprotective effects in a motor neuronal cell line expressing mutant (G93A) human SOD1. This cell line was partially protected from H_2O_2 induced cell death at concentrations of EGCG greater than 20 μM [200]. This compound has also been tested in the G93A mouse model of ALS at doses of 1.5, 2.9 and 5.8 mg/kg, once a day, orally from 60 days of age [201]. At the two higher doses of 2.9 and 5.8 mg/kg, a significant extension in survival was seen with an increase in mean survival of 19 and 20 days respectively.

12. Prospects for the future

Despite the lack of success so far in ALS, it is clear that few potent anti-oxidants able to target the CNS have been tested in trials powered to detect an effect on survival of less than 50%. However, there is hope for the future and some of the promising ways forward are outlined below. Firstly, identification of more potent and less toxic inducers of the ARE response outlined above could prove useful agents in ALS. For example, EGCG is able to induce the Nrf2-ARE pathway probably because it is itself pro-oxidant [202] substantially reducing its therapeutic window. This is likely to be true for other molecules which are known to activate the ARE. A more refined search for compounds with this activity but with minimal toxic effects and their subsequent testing in pre-clinical models of ALS may prove worthwhile. Secondly, the mitochondrial targeting of compounds with anti-oxidant properties is also an interesting possibility. As described earlier, mitochondria are a major source and target of ROS within motor neurons and it would be of therapeutic benefit to be able to target anti-oxidants to this compartment. A lipophilic triphenylphosphonium cation when conjugated to co-enzyme Q10 [203] or vitamin E [204] was shown to target these molecules to mitochondria and the conjugates were several hundredfold more active in mitochondrial oxidative-stress induced cell death assays [204] than the unconjugated molecules. Such an approach could be of great benefit in vivo, particularly if conjugation also enhances CNS penetrance of these molecules. Finally, of the approaches already in the clinic, AEOL10150 is probably the most promising given the relatively large effects in the G93A mouse model and its potential to be fast-tracked through the development process.

It is worth noting that despite the cost, time and difficulty involved in running ALS trials in an academic setting, there are several examples of well-conducted trials which have given useful insights. In order to develop effective drugs which target these pathways in ALS patients and conduct true translational research, however, a much greater collaborative effort is required and every stage of the development process needs to be optimised by those best placed to do so. This is particularly important in view of the fact that few pharmaceutical/biotech R&D programmes are active in this small and difficult field. We believe this review provides a compelling case for targeting oxidative stress in ALS, an international collaboration of like-minded academic and industrial scientists, clinicians and government agencies is now needed to see this exciting project through to fruition.

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