Research article

Different roles of radical scavengers – ascorbate and urate in the cerebrospinal fluid of amyotrophic lateral sclerosis patients

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Ferrous iron, released from iron deposits in the motor cortex and other brain regions of amyotrophic lateral sclerosis (ALS) patients, participates in the Fenton reaction in cerebrospinal fluid (CSF) alongside H_2O_2, which is continuously released by neuronal cells. *In vivo*, the production of notoriously reactive hydroxyl radicals via this reaction could lead to the progression of the disease. Herein, we have examined the effect of ascorbate and uric acid on the production of hydroxyl radicals in CSF from both sporadic ALS patients and control subjects. Electron paramagnetic resonance spectroscopy identified ascorbyl radicals in CSF from ALS patients whereas it was undetectable in control CSF. The addition of H_2O_2 to the CSF from ALS patients provoked further formation of ascorbyl radicals and the formation of hydroxyl radicals *ex vivo*. The hydroxyl addition of uric acid to CSF from ALS patients diminished the production of hydroxyl radicals. In conclusion, there are clear differences between the roles of the two examined radical scavengers in the CSF of ALS patients indicating that the use of ascorbate could have unfavourable effects in ALS patients.

Keywords: amyotrophic lateral sclerosis, iron, EPR, ascorbate, urate

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease resulting from degeneration of cortical, bulbar and spinal motor neurons. ALS is clinically characterised by progressive weakness, muscle atrophy, spasticity, fasciculation and cramps. Patients usually die from paralysis of respiratory and bulbar muscles within 3–5 years after disease onset.[1] At the time when the symptoms and clinical signs of ALS are evident, a large number of molecular and cellular processes are disturbed within motor neurons. These include mitochondrial[2] and cytoskeletal[3] malfunctions, irregular metabolism of glutamate[4] and

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Abbreviations: CSF, cerebrospinal fluid; ALS, amyotrophic lateral sclerosis; EPR, electron paramagnetic resonance; DEPMPO, 5-diethoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide; $HO^{\bullet}$, hydroxyl radical

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disturbed metabolism of reactive oxygen and nitrogen species.[5] All of these pathological processes participate in motor neuron cell death via mechanisms that promote each other mutually, an example being the relationship between excitotoxicity and reactive oxygen species which is supported by the demonstration that free radicals can be generated in neurons exposed to excitatory amino acids[6] and that excitotoxicity can be increased by oxidative stress.[7]

It seems that oxidative stress plays a key role in neuronal death related to ALS[8] as oxidative damage has been observed in the nucleus,[9] membranes,[10] mitochondria,[11] endoplasmic reticulum[12] and Golgi complex[13] of neurons in ALS. As in other neurodegenerative diseases, such as Parkinson's and Alzheimer's diseases,[14] a breakdown in the homeostasis of redox-active iron represents a hallmark of ALS. Deposits of ferric iron in the motor cortex and other brain regions affected in ALS have been observed by several magnetic resonance imaging studies.[15–18] A significant increase in the level of iron in the cortex of Guamanian patients with ALS was also detected.[19] Motor neurons faced with an increased presence of iron seem to protect themselves via increased production of iron-binding proteins such as lactotransferrin, the level of which is increased in the cortex of ALS patients.[20] Finally, we have recently reported that the level of iron in the CSF from ALS patients is slightly increased (0.70 ± 0.05 μM) when compared to CSF from control subjects (0.56 ± 0.07 μM).[21] Nervous tissue spontaneously generates and releases H_2O_2 into the CSF where, even under physiological conditions, its concentration can reach up to 1 mM, while in neurodegeneration H_2O_2 production is promoted via several different pathways.[22,23] The availability of iron to H_2O_2 sets up conditions for Fenton chemistry to develop in CSF, generating $HO^{\bullet}$ radicals and changing oxidative status of CSF. However, the catalytic activity of iron can be affected by various reducing agents present in CSF such as ascorbate and uric acid whose roles in oxidative processes related to neurodegeneration have been examined only briefly.[24–27] *In vitro*, ascorbate and uric acid may act both as antioxidants and pro-oxidants, but which of these contrasting faces each of them show in the CSF from ALS patients and other neurodegenerative conditions is not known.

We propose that ascorbate and uric acid may have different effects on the catalytic activity of iron and related radical production in neurodegeneration. Therefore, in the present study, we have explored both the potential positive and negative effects of ascorbate and uric acid on the oxidative status of the CSF from ALS patients.

Subjects and methods

Experiments with CSF

Sporadic ALS patients were informed that their CSF was to be used for both routine medical analyses and our laboratory research. ALS was diagnosed according to the revised El Escorial criteria[28] with disease duration less than 3 years. The current study was performed using 15 recently diagnosed ALS patients with a clinical status consistent with probable or definite ALS according to El Escorial criteria. Patient recruitment, counselling, sample collection and handling were conducted according to internationally recognised ethical standards (The Helsinki Declaration of 1964, as revised in 1975, 1983 and 1989). Institutional approval for the study was granted by The Clinics Ethics Committee which followed international guidelines. Each study participant provided written consent. The ALS patient group consisted of 10 males and 5 females (mean age, 54.5 ± 8.9 years). The spinal onset of ALS was present in 13 patients, while two demonstrated the bulbar onset. Serving as controls was a group of 15 age- and sex-matched patients with other neurological disorders (2 migraines, 3 tension headaches, 5 lumbar disc herniations and 5 cervical disc herniations). The mean age of this group of patients was 52.8 ± 11.1 years. None of the patients from either group had blood–CSF barrier dysfunction. CSF samples (2 ml) were obtained by lumbar puncture at the point when diagnosis took place and the remainder of the samples (~1 ml) was used for this study. Samples were drawn after an overnight bed rest and fasting. The samples were centrifuged (5000 *g*, 10 min at 4ºC) and rapidly frozen and stored at –80ºC. All patients received a balanced diet prescribed by a nutritionist without any supplementary vitamins.

Reagents

All chemicals were of analytical grade or better: H_2O_2 was purchased from Renal, (Budapest, Hungary); uric acid from Merck (Darmstadt, Germany); and DEPMPO was from Alexis Biochemicals (Lausen, Switzerland). The latter was purified twice in order to remove hydroxylamine impurities that can reside in commercial DEPMPO preparations which when oxidised can represent the origin of unwanted signals.[29]

Biochemical assays

The concentration of ascorbic acid in untreated CSF samples was determined by the α,α'-bipyridyl method described by Okamura[31] using a standard curve prepared with ascorbic acid (10–300 μM). This

method enables the measurement of the concentration of ascorbic acid, not taking into account ascorbyl radical or dehydroascorbate. The concentration of uric acid in untreated CSF samples was determined using a commercial Managent uric acid HF kit (Menarini Diagnostics, Winnersh-Workingham, Berkshire, UK).

EPR spectroscopy

EPR spectroscopy of ascorbyl radicals was performed in untreated CSF samples and samples treated with H_2O_2 (final concentration 1 mM). The production of $HO^{\bullet}$ radical was determined, using EPR spin-trapping spectroscopy with spin-trap DEPMPO (14 mM), in CSF samples supplemented with H_2O_2 (1 mM), with or without pre-added uric acid (30 μM). Samples were placed in Teflon tubes with a wall thickness of 0.025 mm and an internal diameter of 0.6 mm (Zeus Industries, Raritan, NJ, USA) and inserted into quartz capillaries. EPR spectra were recorded at room temperature using a Varian E104-A EPR spectrometer operating at X-band (9.51 GHz), using the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW. All spectra were recorded using EW software (Scientific Software Inc., Bloomington, IL, USA). Computer spectral simulations were used for identification and quantification of intensity (I) of spectra. Simulation of EPR spectrum of ascorbyl radical was performed using the WINEPR SimFonia Computer Program (Bruker Analytische Messtechnik GmbH, Karlsruhe, Germany) and simulation parameter: a^H = 2.3. The simulation of EPR signal of DEPMPO/OH adduct was performed using parameters: a^P = 46.70; a^N = 13.64; a^H = 12.78).[30]

Statistical analysis

Statistical analysis was carried out using Statistica v6.0 (StatSoft Inc., Tulsa, OK, USA). The results are presented as mean values (of at least 10 experiments) ± SD. Statistical significance was determined using a non-parametric two-tailed Mann–Whitney test to compare each pair of data ($P < 0.05$).

Results

The level of ascorbate in untreated CSF samples was significantly lower in ALS patients (96.1 ± 2.6 μM) when compared to controls (127 ± 5.8 μM). In contrast, the level of uric acid was significantly higher in ALS subjects (45.4 ± 2.1 μM) than in control CSF (18.3 ± 0.7 μM).

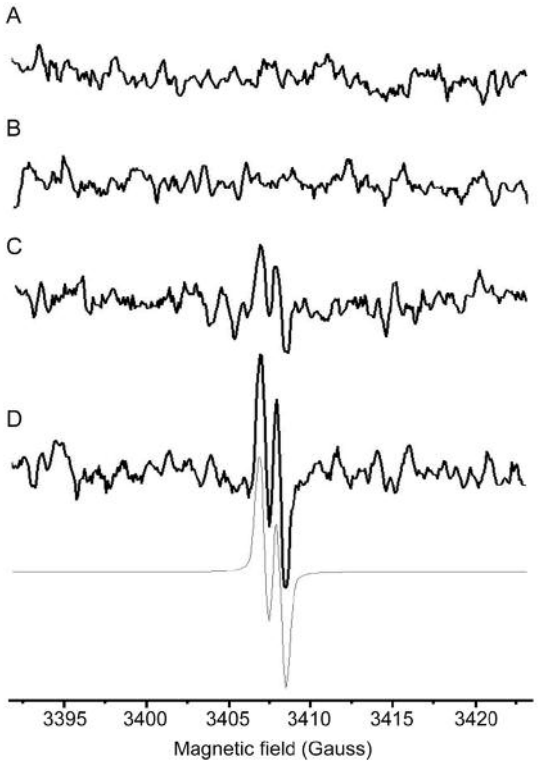

Figure 1 EPR spectra of CSF. (A) Control CSF with no added H_2O_2; (B) Control CSF + H_2O_2 (1 mM); (C) ALS CSF with no added H_2O_2 (I = 46 ± 6); (D) ALS CSF + H_2O_2 (I = 111 ± 10). The grey trace represents spectral simulation of EPR signal of the ascorbyl radical (a^H = 2.3). The presented spectra are from a single sample and are representative of signals obtained from CSF from 15 ALS patients and 15 controls

The ascorbyl radical was not detected in CSF from control subjects either before (Fig. 1A) or after (Fig. 1B) supplementation with H_2O_2. In contrast, the ascorbyl radical was clearly present in the CSF isolated from ALS patients (Fig. 1C). The addition of H_2O_2 led to an increase in the level of the ascorbyl radical (Fig. 1D).

The addition of H_2O_2 provoked some production of $HO^{\bullet}$ radicals in control samples but it was below the detection limit of the method (Fig. 2A). In contrast, significant production of $HO^{\bullet}$ radicals in CSF from ALS patients was clearly driven by H_2O_2 (Fig. 2B). The addition of uric acid prior to the supplementation of H_2O_2 diminished the formation of $HO^{\bullet}$ radicals in the CSF from ALS patients (Fig. 2C).

Discussion

Oxidative stress has been proposed to represent a key player in the progress of neuronal damage related to

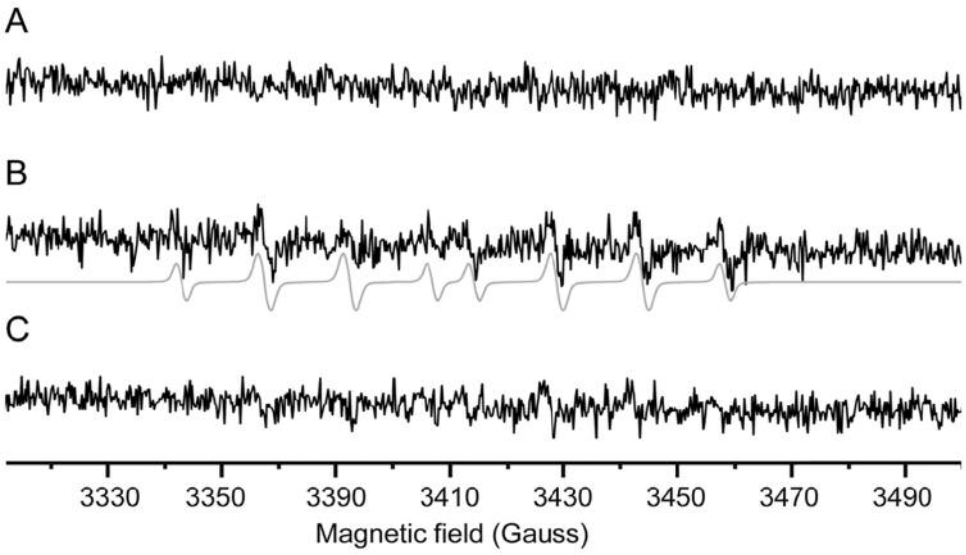

Figure 2 Characteristic EPR spectra of the DEPMPO/OH adduct in: (A) Control CSF + H_2O_2 (1 mM); (B) ALS CSF + H_2O_2 (I = 52 ± 4); (C) ALS CSF + uric acid (30 μM) + H_2O_2 (1 mM) (I = 22 ± 3). Grey – spectral simulation of EPR spectrum of DEPMPO/OH. The central two lines in the spectrum of DEPMPO/OH adduct in (B) overlap the ascorbyl radical signal

ALS.[33] Increased ascorbyl radicals (Fig. 1C), which represents a marker of oxidative status,[34] in the CSF of ALS patients verifies previous indications[21] and clearly demonstrates that the CSF is exposed to oxidative stress in ALS conditions. A lower *in vivo* level of ascorbic acid in the CSF from ALS patients when compared to controls is provoked by transformation of ascorbate into ascorbyl radicals which verifies the EPR results. We noticed in a previous study[21] that the CSF of ALS patients has a higher level of iron related to deposits of ferric iron in the motor cortex which is in contact with the CSF.[15–17] Ascorbyl radicals may develop in the presence of an increased level of iron in CSF and brain cortex via two reactions related to two different roles of ascorbate – pro-oxidative (reaction 1) and antioxidative (reaction 3):[34]

$$Fe^{3+} + Asc \rightarrow Fe^{2+} + Asc^{\bullet} \qquad \text{Eq. 1}$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^{-} \qquad \text{Eq. 2}$$

$$Asc + HO^{\bullet} \rightarrow Asc^{\bullet} + OH^{-} \qquad \text{Eq. 3}$$

Another ascorbyl radical could be involved in this process generating one ascorbate and one dehydroascorbate.[35] Therefore, a lower level of ascorbate in parallel with a higher level of ascorbyl radicals in the CSF isolated from ALS patients (compared with controls; Fig. 1C) may arise via: (i) ascorbate's reaction with ferric iron in deposits which leads to a reduction of the ferrous form and iron release (this process may be similar to Fe^{3+} reduction to Fe^{2+} by ascorbate which facilitates iron uptake in the duodenum[36]); and (ii) increased generation of $HO^{\bullet}$ radicals which are then scavenged by ascorbate generating ascorbyl radicals. Ferric iron, another product of the Fenton reaction, readily forms complexes with hydroxyl and phosphate ions present in CSF and can be redeposited into the cortex which closes pro-oxidative loop within the CSF (Fig. 3). It should be stressed that ferric iron is able to catalyse the oxidation of ascorbic acid with concomitant formation of H_2O_2, further increasing the level of H_2O_2.[37] Also, the pro-oxidative loop driven by copper-mediated redox transformation,[38] cannot be excluded in the case of ALS. From our results, we conclude that ascorbic acid is transformed into ascorbyl radicals in CSF of ALS patients in a manner that increases the catalytic activity of the present metals thus promoting $HO^{\bullet}$ radical production through the Fenton reaction. Halliwell and Gutteridge[36] proposed that, if iron from intracellular 'pools' come into the contact with extracellular ascorbate, pro-oxidant effects could conceivably occur and they hypothesised that 'giving lots of ascorbate to sick people may not be a good thing'. This may be particularly true for neuro-degenerative conditions. Ascorbate is a vital molecule for brain function, acting as neuromodulator, co-factor of enzymes and modulator of metabolism.[39,40] As such, ascorbate is actively transported to the central nervous system where it is accumulated at concentrations several-fold higher than in plasma.[39,40] Therefore, the pro-oxidant effects of ascorbate related to transition metals may be amplified in CSF when compared to other liquids and tissues as CSF accumulates ascorbate,

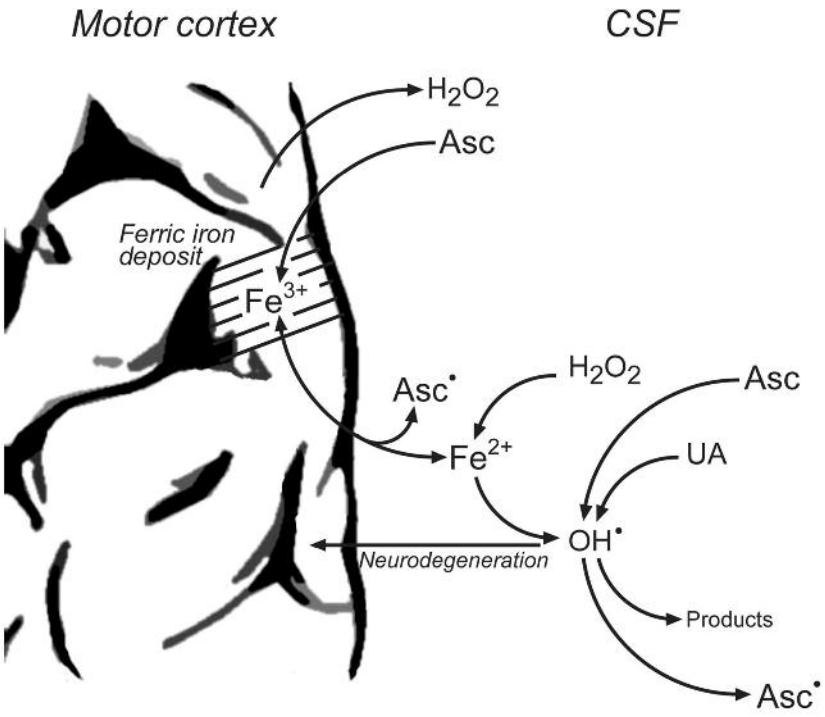

Figure 3 Possible roles of ascorbate and uric acid (UA) in the CSF from ALS patients. Ascorbate reduces Fe^{3+} within iron deposits in the brain of ALS patients which leads to Fe^{2+} release. Fe^{2+} reacts with H_2O_2 to give $HO^{\cdot}$ and Fe^{3+} which may be redeposited in the cortex. Some $HO^{\cdot}$ radicals are removed by uric acid and ascorbate but the remainder provoke oxidative damage

but also because the CSF metal-binding antioxidative defence is weak containing little transferrin, albumin and ceruloplasmin.[36] Despite the fact that ascorbate demonstrated its antioxidative properties by scavenging $HO^{\bullet}$ radicals (Eq. 3) in the CSF supplemented with H_2O_2 *ex vivo* (Fig. 1D), the administration of vitamin C may actually be detrimental to patients with ALS. Pertinent to this, it seems that caution should be exercised when using antioxidant therapies in general.[36] It should be stressed that the ascorbate in the CSF is not detrimental by itself (control CSF contains higher level of ascorbate than ALS samples) but may become a problem when combined with misbalanced metabolism of iron as in ALS.

Uric acid is an important antioxidant in the body and a strong peroxynitite scavenger.[41,42] We showed herein that the level of uric acid in CSF from ALS patients is significantly higher when compared to controls. Such an increase may arise from breakdown of DNA and RNA purines in necrotic and apoptotic cells and breakdown of ATP and adenosine.[43] However, it could also represent part of the intrinsic defence system against disturbed oxidative status in CSF as uric acid showed high $HO^{\bullet}$ scavenging capacity (Fig. 2). Pertinent to this, Stover and colleagues[27] noted a 2–3-fold increase in uric acid in the CSF from patients with many neurological disorders such as myelopathy, stroke, epilepsy and viral meningitis. Uric acid's precursor inosine has been used in the treatment of multiple sclerosis and other neurodegenerative diseases with some positive effects.[44] It appears from our results that uric acid predominantly acts as antioxidant in the CSF.

Conclusions

There are clear differences in the role of the two examined radical scavengers in the CSF of ALS patients (Fig. 3) suggesting that the use of ascorbate could have unfavourable effects in ALS patients or at least it could be futile.

Acknowledgements

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