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Increase in oxidized NO products and reduction in oxidized glutathione in cerebrospinal fluid from patients with sporadic form of amyotrophic lateral sclerosis

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Abstract

To determine the role of free radical mechanisms in the pathogenesis of amyotrophic lateral sclerosis (ALS), cerebrospinal fluid concentrations of oxidized nitric oxide (NO) products (nitrite and nitrate) and reduced and oxidized forms of glutathione (GSH and GSSG, respectively) were compared between patients with the sporadic form of ALS (SALS) and controls. In the SALS patients, the nitrate levels were significantly higher (by 73%) in contrast to remarkably lower GSSG/GSH ratio, approximately 3-fold, compared to controls. These results suggest that NO production or oxidation is activated in SALS patients, leading to a decrease in superoxide radicals to oxidize GSH. The subsequent generation of a highly reactive anion, peroxynitrite, may play a causal role in the pathogenesis of SALS.

Keywords: Amyotrophic lateral sclerosis; Nitric oxide; Glutathione

Amyotrophic lateral sclerosis (ALS) is a progressive, fatal disease characterized by the selective degeneration of upper and lower motor neurons, leading to death usually within 5 years. Approximately 5–10% of ALS is familial (FALS) with the remaining 90–95% arising sporadically (SALS). Although still unknown, the pathogenesis of ALS is hypothesized to involve free radical injury [3], glutamate-mediate excitotoxicity [10], or cytoskeletal abnormalities [7–9]. The recent discovery of point mutations and a small deletion in the Cu/Zn superoxide dismutase gene (SOD1) on chromosome 21 in some FALS and SALS [1,15,20,21,23] supports the free radical hypothesis for the pathogenesis of ALS. The enzymatic activity of SOD1 to scavenge superoxide radicals generally decreases upon genetic mutation. However, this activity is unaltered in some FALS patients [1,5] and even increases in transgenic mice [11]. In SALS, reduced [19,23] or normal [6] SOD1 activity and increased SOD1 mRNA levels [4] in the central nervous system (CNS) have been reported. Free radicals are reduced by scavengers such as glutathione (GSH) and α-tocopherol, which are themselves oxidized to the oxidized form of glutathione (GSSG) and α-tocopherol quinone, respectively. Free radical production is also linked to the excitotoxicity hypothesis [10]. In this model, stimulation of glutamate-mediated neurotransmission causes excessive Ca^{2+} influx and nitric oxide (NO) production, and resultant overproduced NO is oxidized to a highly reactive anion, peroxynitrite ($ONOO^-$), leading to neuronal damage [3]. Peroxynitrite is in turn metabolized to the more stable oxidation products nitrite (NO_2^-) and nitrate (NO_3^-). To investigate the role of free radicals in the pathogenesis SALS, we for the first time determined concentrations of oxidized NO products, NO_2^- and NO_3^-, and reduced (GSH) and oxidized (GSSG) forms of glutathione, a free radical scavenger, in the cerebrospinal fluid (CSF) of SALS patients.

Throughout this study, SALS was diagnosed based on neurological history, neurological examination, and laboratory tests [17]. The severity of the disease was evaluated using the ALS index developed by Jablecki et al. [14], which is derived by adding disability scores in 12 areas, with a maximum score of 40. Controls were neurologically normal patients who underwent lumbar anesthesia for minor

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surgery. The SALS patients and controls used for analysis of NO_2^-/NO_3^- and GSH/GSSG were different. Nitrite and nitrate were determined in 18 SALS patients (60 ± 13 years old) with a disease duration of 1.2 ± 0.9 years and an ALS score of 13.9 ± 4.5, and in 14 controls (62 ± 11 years old). GSH and GSSG were determined in 12 SALS patients (61 ± 8 years old) with a disease duration of 0.9 ± 0.6 years and an ALS score of 11.7 ± 5.4, and in 15 controls (68 ± 5 years old). All patients were admitted to the hospital and were administered a standard diet. Informed consent was obtained from all patients. Lumbar CSF was obtained with the patients in the lateral decubitus position between 09:00 and 10:00 h after overnight bed-rest and fasting. The CSF samples drawn from the patients were rapidly frozen and stored at −80°C for 1 month to 2 years.

The nitrite and nitrate concentrations were determined according to Yamada and Nabeshima (1997) [24]. A 20 μl aliquot of each CSF sample was injected into an automated NO detector-HPLC system (ENO-10; Eicom, Kyoto, Japan). NO_2^- and NO_3^- were separated using a reverse-phase separation column (NO-PAK, 4.6 × 50 mm; Eicom, Kyoto, Japan), and NO_3^- was reduced to NO_2^- in a reduction column (NO-RED; Eicom, Kyoto, Japan). NO_2^- was mixed with Griess reagent to form a purple azo dye in a reaction coil. The separation and reduction columns and the reaction coil were maintained at 35°C. The product dye absorbance at 540 nm was measured using a flow-through spectrophotometer (NOD-10; Eicom, Kyoto, Japan). The mobile phase (10% methanol containing 0.15 M NaCl/NH_4Cl and 0.5 g/l 4 Na-EDTA) was delivered at a rate of 0.33 ml/min; the Griess reagent (1.25% HCl containing 5 g/l sulfanilamide with 0.25 g/l N-naphthylethylenediamine) was delivered at a rate of 0.1 ml/min. The detection limit of both NO_2^- and NO_3^- was 10 nM. $NaNO_2$ and $NaNO_3$ were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Simultaneous determination of GSH and GSSG was performed as described previously by Harvey et al. [12]. Briefly, a 500-μl sample of the CSF was mixed with 500 μl of 0.6 N perchloric acid containing 1 mM EDTA, and centrifuged at 3000 rev./min for 10 min. A 500-μl aliquot of supernatant was stored at −80°C. A 100 μl aliquot of each sample was analyzed using an HPLC system (MCM C18 reversed phase column, 250 × 4.6 mm, MC medical, Tokyo, Japan) equipped with an electrochemical detector (Model 5,100 A, ESA, Bedford, MA, USA). The electrode potentials were maintained at 0.75 V for the guard cell, 0.4 V for the conditioning cell, 0.6 V for detector I (for GSH), and 0.7 V for detector II (for GSSG). The mobile phase consisted of 0.01 M sodium perchlorate monohydrate containing 0.15 mM sodium octanesulfonic acid (pH 2.7), and acetonitrile (98:2). The detection limit of both GSH and GSSG was 100 pM. GSH and GSSG were obtained from Sigma Chemical (St. Louis, MO, USA).

While nitrite levels were unaltered, nitrate levels were significantly higher in SALS patients (by 73%, $P < 0.01$) (Fig. 1A,B), suggesting overproduction of NO. The GSH level was significantly higher (by 102%, $P < 0.001$), and the GSSG level was significantly lower (by 33%, $P < 0.01$) in the SALS patients than in the controls, with a significantly lower (3-fold) GSSG/GSH ratio in the SALS patients than controls ($P < 0.001$) (Fig. 1C,D).

The present results demonstrate a significant increase in the nitrate concentration and a remarkable reduction in the GSSG/GSH ratio in the CSF of SALS patients. The mechanisms by which NO production increases in SALS are unknown. Although the possibility that the increased nitrate levels may be in part accounted for by increased catabolism, they are most likely glutamate-mediated [3,10] in the context of currently proposed hypotheses for the pathogenesis of ALS. The findings on GSH and GSSG are very similar to those of our previous report in which the ratio of α-tocopherol quinone (α-TQ) to α-tocopherol (α-TOH) concentrations remarkably decreased in the CSF from patients with SALS [22]. The interpretation of these findings is somewhat difficult. However, superoxide reacts with NO to form the

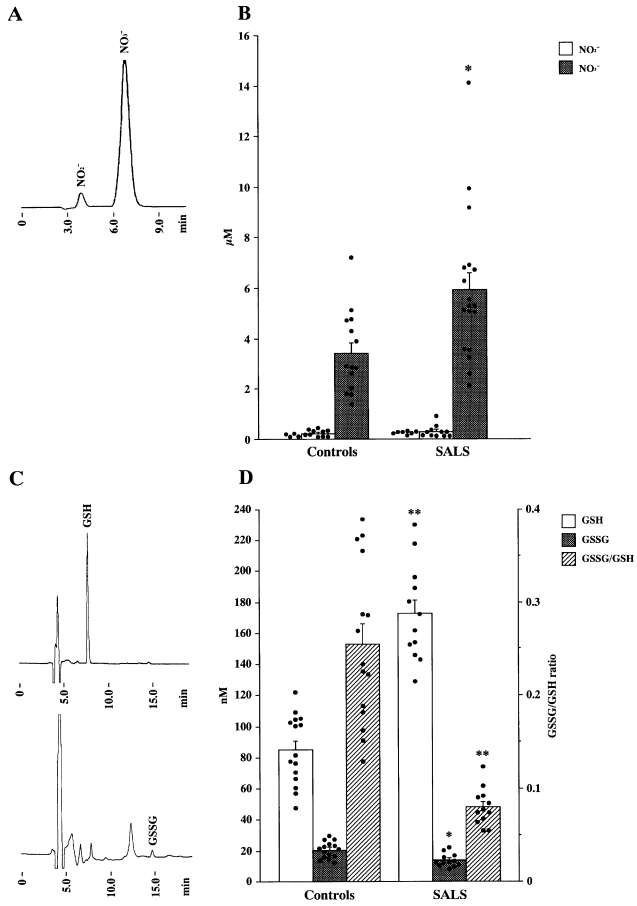

Fig. 1. Typical chromatograms of cerebrospinal fluid samples for glutathione (GSH) and oxidized glutathione (GSSG) in (A) and nitrite and nitrate in (C). Concentrations of GSH and GSSG (B) and nitrite and nitrate (D) with means (columns) and standard errors (vertical bars) are compared between controls and patients with the sporadic form of amyotrophic lateral sclerosis (SALS). In (D), the left ordinate scales indicate concentrations, and the right ordinate scales indicate the ratio of GSSG/GSH. *$P < 0.01$, **$P < 0.001$ compared with controls using Mann–Whitney test.

powerful oxidant, peroxynitrite ($ONOO^-$), 3-fold faster (6.7×10^9 $M^{-1}s^{-1}$) than SOD1 (2×10^9 $M^{-1}s^{-1}$) [13,16], and 10 000-fold faster than GSH (6.7×10^5 $M^{-1}s^{-1}$), α-TOH (5×10^5 $M^{-1}s^{-1}$), and ascorbic acid ($5–27 \times 10^4$ $M^{-1}s^{-1}$) [2,18]. Moreover, NO ($\approx \mu$M) in the CSF is approximately 1000-times more abundant than either GSH or α-TOH ($\approx$ nM). Therefore, one possibility may be that oxidation of overproduced NO may greatly surpass oxidation of GSH and α-TOH, which result in the reduction in the generation of GSSG and α-TQ.

In conclusion, the concentration of oxidative NO products is significantly higher and that of the oxidized form of a free radical scavenger GSSG is markedly lower in the CSF of SALS patients, suggesting activated NO production or oxidation. These findings support the hypothesis that glutamate-mediated overproduction of NO and subsequent generation of peroxynitrite play an important role in the pathogenesis of SALS.

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