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ORIGINAL ARTICLE

Erythropoietin and amyotrophic lateral sclerosis: Plasma level determination

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Abstract
In amyotrophic lateral sclerosis (ALS), respiratory muscle weakness causes ventilatory insufficiency and tissue hypoxia, which induces a number of metabolic pathways, and in particular increases erythropoietin (EPO) synthesis. EPO is a glycoprotein with neuroprotective properties that stimulates erythropoiesis. Here, EPO plasma level in a large population of ALS patients, with and without respiratory failure, was measured. Plasma EPO level of patients with ALS (n = 98), controls with other neuromuscular diseases (n = 58) and healthy controls (n = 20) has been quantified by ELISA. No significant difference was found between ALS patients and the two control groups. EPO level was not different between bulbar- and spinal-onset patients and was not correlated with disease duration or functional impairment. However, in the ALS group EPO level was higher in females (p = 0.0006) and correlated positively with age (p = 0.006). The subgroup of ALS patients with respiratory failure had higher plasma levels of EPO compared with ALS patients with preserved respiratory function (p = 0.016), but short-term non-invasive ventilation did not change EPO level. In conclusion, EPO levels were found to be significantly higher in ALS patients with respiratory impairment representing preservation of this homeostatic mechanism.

Key words: Amyotrophic lateral sclerosis, erythropoietin, plasma, phrenic nerve, respiratory failure

Introduction
Amyotrophic lateral sclerosis (ALS) is a rapidly progressive neurodegenerative disease characterized by degeneration of motor neurons in the motor cortex, brainstem and spinal cord causing progressive muscle weakness, in particular respiratory muscle weakness and death, typically within 3–5 years after onset (1).

Respiratory failure is mainly due to severe diaphragm weakness (2), which results in tissue hypoxia (3). The main mechanism of cellular adaptation to low oxygen tension is the activation of hypoxia-inducible factor-1 (HIF-1), which leads to the transcriptional induction of several genes, including erythropoietin (EPO) and vascular endothelial growth factor genes that participate in erythropoiesis, angiogenesis, iron and glucose metabolism, and cell proliferation/survival (4). In addition, the hypoxia-inducible factor-2α (HIF-2α) pathway appears to play a key role in regulating EPO production and erythropoiesis in adult mouse whereas HIF-1α is also required for yolk sac erythropoiesis, renal EPO production in response to acute hypoxia and the development of polycythemia in response to chronic hypoxia (5).

EPO is an approximately 30 kDa glycoprotein that is a member of the type I cytokine superfamily. EPO maintains tissue oxygenation by regulating erythrocyte differentiation in the bone marrow. Tissue hypoxia might cause the EPO serum level to increase by 50-fold (6). Besides regulating erythropoiesis, EPO has neuroprotective effects following the ischaemic, hypoxic, metabolic, neurotoxic or excitotoxic insults to the central nervous system (CNS) (7). Indeed, EPO and its receptor are expressed in the CNS, and peripherally-administered...
EPO has been shown to cross the blood-brain barrier in neuroprotective concentrations. The EPO tissue protective-receptor has been proposed to be distinct from the EPO erythroid-receptor (6).

Pre-clinical trials with EPO have shown some beneficial effects in animal models of ALS, but this requires further investigation (7). In humans, clinical trials have taken place in stroke, schizophrenic and multiple sclerosis (8). EPO administration to ALS patients was found to be safe and well tolerated; however, it was not possible to draw conclusions about its efficacy (9). Additionally, a clinical trial with granulocyte-stimulating factor and EPO to treat ALS patients was initiated (http://clinicaltrialsfeeds.org/clinical-trials/show/NCT00298597); however, the outcome is not yet known.

In the literature, the existing reports concerning blood EPO level in ALS are contradictory. Decreased level was reported in a few studies (10,11), and in one the EPO reduction was statistically significant (12). Other authors described a non-significant increase of the plasma level in hypoxaemic ALS patients (13). In the CSF, significantly reduced level of EPO was detected by some authors (10–12), whereas other groups observed a significant increase in patients with hypoxia (13).

In the present study, we tested the plasma EPO level in a large group of ALS patients, including a subgroup of patients with marked respiratory failure who were promptly submitted to non-invasive ventilation.

Materials and methods

All patients included were regularly followed at the Neuromuscular Unit of the Department of Neurosciences (Hospital de Santa Maria, Lisbon). ALS patients had probable or definite disease and showed disease progression, according to the revised El Escorial criteria (14). As inclusion criteria we established age between 18 and 90 years, and informed consent. We excluded patients with other medical conditions such as diabetes, oncologic disease, heart insufficiency or lung disorders. At the time of blood sampling the patients were observed and the disease severity was scored applying ALSFRS (Amyotrophic Lateral Sclerosis Functional Rating Scale) (17); forced vital capacity was determined at the same time using a methodology previously described elsewhere (2).

We included 98 ALS patients, 58 controls with other neurological diseases and 20 healthy controls. The healthy group (H) consisted of the patients' family members or volunteers working at the hospital. The group with other neurological diseases (OD) incorporated adult patients with Charcot-Marie Tooth disease (types I and II), familial amyloid polyneuropathy, muscle dystrophies (facioscapulohumeral muscular dystrophy, myotonic muscular dystrophy, sargoglycanopathy, dystrophinopathy), Machado Joseph disease, spinal muscular atrophy type III and spastic paraparesis, who accepted to participate. No patient in the group OD had respiratory symptoms. No patient in any group was on tube feeding or taking any nutritional supplement other than vitamins. Informed consent was obtained from all subjects and the research protocol was approved by the local ethics committee.

Blood was collected in a tube containing ethylenediaminetetra-acetic acid, centrifuged at 1000 × g for 10 min, and the supernatant consisting of plasma was conserved at −80°C. EPO level in plasma was determined with an enzyme-linked immunosorbenet method using commercial ELISA assay (Quantikine®; R&D Systems) in accordance with the manufacturer's instructions. The range of EPO concentrations in the plasma and serum of healthy individuals are 3.1–14.9 miU/ml and 3.3–16.6 miU/ml, respectively (Quantikine IVD kit manual).

Data analysis was performed using Excel or GraphPad Prism® (GraphPad Software, Inc.). We applied D’Agostino & Pearson omnibus normality test to test data distribution and percentiles and the interquartile range of the data to classify outliers (15).

One-way ANOVA was used to compare the three groups. The unpaired t-test, assuming equal variances, was applied to test differences between the two subgroups in the ALS population. A linear regression model was employed to describe the relationship between tested variables. p-values < 0.05 were considered as statistically significant.

Results

EPO plasma level showed a normal distribution in the three groups, but it was non-significantly higher in the ALS group (8.52 ± 3.76 miU/ml) (Table I) compared with the two other tested groups (p = 0.35). In ALS patients the EPO level was higher in females than in males (p = 0.0006) (Table I); this gender difference was not observed in the other groups. We found a significant positive correlation of EPO levels with age in the ALS group (R = 0.28, p = 0.0006), but this was not verified in the other diseases and healthy control groups (R = 0.19, p = 0.18; and R = 0.24, p = 0.35, respectively). However, in the ALS group, there was no correlation between EPO level and disease duration (R = 0.03, p = 0.80) or with ALSFRS (R = 0.18, p = 0.1) or ALSFRS-R (R = 0.18, p = 0.11). In addition, EPO levels were not different between bulbar onset (n = 30) and spinal onset (n = 66) patients (p = 0.56). A subgroup of six ALS patients were monitored over disease progression (mean of 1.1 years); we observed a non-significant EPO level increase (p = 0.47).

We tested a group of 24 patients with severe clinical respiratory impairment at the time of our first evaluation, with abnormal respiratory subscore on ALSFRS-R (less than 10, maximum 12), low FVC, marked hypoxia on nocturnal percutaneous
Erythropoetin and ALS

Table I. Demographic information of study population and EPO plasma concentrations in subgroups.

<table>
<thead>
<tr>
<th></th>
<th>Number</th>
<th>Age</th>
<th>Disease duration</th>
<th>Respiratory subscore</th>
<th>FVC</th>
<th>Mean O₂ on PNO</th>
<th>EPO level</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>98</td>
<td>60.9 ± 14.5</td>
<td>2.9 ± 3.2</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>8.52 ± 3.76</td>
</tr>
<tr>
<td>Males</td>
<td>52</td>
<td>58.9 ± 15.9</td>
<td>2.3 ± 1.9</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>7.33 ± 2.77**</td>
</tr>
<tr>
<td>Females</td>
<td>46</td>
<td>63.2 ± 12.5</td>
<td>3.6 ± 4.1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>9.87 ± 4.27**</td>
</tr>
<tr>
<td>Bulbar</td>
<td>30</td>
<td>60.4 ± 13.6</td>
<td>2.0 ± 1.7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>8.88 ± 3.82</td>
</tr>
<tr>
<td>Spinal</td>
<td>68</td>
<td>58.4 ± 14.3</td>
<td>3.5 ± 3.6</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>8.39 ± 3.78</td>
</tr>
<tr>
<td>RI</td>
<td>24</td>
<td>69.4 ± 14.1</td>
<td>2.4 ± 2.7</td>
<td>8.46 ± 1.25°</td>
<td>65.50 ± 12.64°</td>
<td>91.06 ± 5.44°</td>
<td>10.11 ± 6.62***</td>
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<tr>
<td>nRI</td>
<td>74</td>
<td>58.1 ± 13.6</td>
<td>3.1 ± 3.3</td>
<td>11.49 ± 0.64°</td>
<td>90.68 ± 16.02°</td>
<td>96.19 ± 1.51°</td>
<td>8.01 ± 3.30°</td>
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<tr>
<td>Controls</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OD</td>
<td>58</td>
<td>54.8 ± 16.6</td>
<td>10.1 ± 10.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>8.78 ± 3.87</td>
</tr>
<tr>
<td>Males</td>
<td>33</td>
<td>57.7 ± 16.1</td>
<td>8.8 ± 9.7</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>8.72 ± 4.23</td>
</tr>
<tr>
<td>Females</td>
<td>25</td>
<td>51.7 ± 7.1</td>
<td>11.4 ± 10.3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>8.86 ± 3.41</td>
</tr>
<tr>
<td>H</td>
<td>20</td>
<td>56.3 ± 11.3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>7.40 ± 2.21†</td>
</tr>
<tr>
<td>Males</td>
<td>7</td>
<td>56.4 ± 12.4</td>
<td>–</td>
<td>–</td>
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<td>–</td>
<td>8.48 ± 2.00</td>
</tr>
<tr>
<td>Females</td>
<td>13</td>
<td>56.2 ± 11.4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>6.83 ± 2.17</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD.
ALS: amyotrophic lateral sclerosis; RI: ALS patients with critical clinical signs of respiratory impairment; nRI: ALS patients without clinical signs of respiratory impairment; OD: controls with other diseases; H: healthy controls; Respiratory subscore: as evaluated using the three questions on respiratory function extracted from ALSFRS-R; FVC: forced vital capacity; mean O₂ on PNO: mean oxygen saturation value on percutaneous nocturnal oximetry; EPO: erythropoietin plasma level.
°Indicates a significant difference between ALS patients with respiratory insufficiency, and controls with other diseases (OD) and healthy (H) controls (p < 0.001, for all comparisons).
*Indicates a significant difference between male and female ALS patients (p < 0.001).
**Indicates a significant difference between ALS patients with respiratory insufficiency and ALS patients with no respiratory insufficiency (p = 0.016).
†Indicates a significant difference between ALS patients with respiratory insufficiency and H controls (p = 0.021).

oximetry and absent or very small motor response on phrenic nerve stimulation (Table I). EPO level in this subgroup was significantly higher (10.11 ± 6.62 mIU/ml) than the value observed in the subgroup of ALS patients without respiratory symptoms (n = 74) (8.01 ± 3.30 mIU/ml; p = 0.016) (Table I). Furthermore, it was significantly higher than the value observed for healthy controls (7.40 ± 2.21 mIU/ml; p = 0.021) (Table I). As shown in Figure 1, we noticed that three patients with critical respiratory impairment had particularly high EPO values, but they were not classified as outliers when applying interquartile range distribution (15). EPO level was re-evaluated in this same population after correction of hypoxia by non-invasive ventilation as evaluated by percutaneous nocturnal oximetry (ranging from 0.5 to 2.3, months later). We did not find a significant change for EPO level (p = 0.69).

Discussion

In this work we investigated EPO levels in a large population of ALS patients, including a subgroup of patients with marked respiratory symptoms requiring immediate non-invasive ventilation. The most relevant finding was a significantly higher EPO level in this subgroup of ALS patients with respiratory insufficiency (Table I). Other authors have described a trend to increased EPO level in ALS patients with hypoxaemia (13). On the other hand, our total population of ALS patients, or the subgroup of ALS patients without respiratory symptoms, did not show a reduced level of EPO, as reported elsewhere (12). The subgroup of ALS patients with respiratory impairment suffered from hypoxia, as confirmed by nocturnal percutaneous oximetry. There is a large body of evidence showing that EPO is produced by the kidney and it is regulated as response to tissue hypoxia (6). Therefore, the increased levels of plasma EPO observed in the hypoxaemic ALS patients were probably due to a normal adaptation…

Figure 1. EPO plasma concentration in ALS patients with respiratory insufficiency, ALS patients with no respiratory insufficiency, and controls with other diseases (OD) and healthy controls (H). The group of patients with ALS and respiratory insufficiency has a mean value of EPO levels significantly higher than the group of ALS patients without respiratory failure (p = 0.016). The two subgroups were compared using the unpaired t-test assuming equal variances.
mechanism as response to hypoxia, and the hypoxia-dependent gene regulation pathway functions correctly in ALS patients. This hypothesis has been previously suggested by Just et al. (13) who found a trend towards an increase in the plasma of hypoxemic patients. Those authors also showed a significant increase in the CSF EPO levels for the hypoxaemic patients, which indicated a similar response to hypoxia in the CNS since different reports suggested that most CSF EPO is produced locally (10,13).

More specifically, EPO levels have been found to be significantly increased in the CSF of ALS patients with chronic hypoxemia, late in the course of the disease (16). The induction has been proposed to occur via the HIF-2 pathway. In the same study other angiogenic and inflammatory factors were examined and had distinct responses: vascular endothelial growth factor and angiogenin were not up-regulated in hypoxemic conditions in ALS patients, in contrast to controls, whereas angiopoietin-2 and prostaglandin-E2 were up-regulated in ALS patients as well as in controls. Therefore, the regulation of these factors is complex and it has been suggested to depend on the regulation and interplay between different regulatory pathways, namely the HIF-1α, HIF-2 and NF-κB pathways (16).

We showed that in hypoxaemic ALS patients EPO remained elevated even after short-term hypoxia correction by non-invasive ventilation. Embury et al. (17) calculated the half-life of endogenous EPO from 1.5 to 2.9 h in anaemic sickle-cell patients whose elevated plasma EPO values went down to normal within two days during therapeutic inhalation of pure oxygen. Most of our patients used NIV at night, remaining free of NIV most hours during the day. The efficacy of nocturnal NIV was confirmed by correction of O2 desaturation, as evaluated by nocturnal percutaneous oximetry. However, the persistent low level of arterial O2 over the day was probably enough to maintain a high level of EPO in the plasma.

In this study we verified that in the ALS population EPO level was not influenced by disease duration, functional impairment as evaluated by ALSFRS, or region of onset. On the other hand the EPO levels were positively correlated with age, as described before for CSF EPO concentration by Brettschneider et al. (10). Moreover, we found that EPO was increased in females with ALS; this gender difference was not confirmed in the other two groups. There are several sites for EPO production in the body: kidney and brain, which are hypoxia inducible; uterus, which is oestrogen and hypoxia/oestrogen inducible; and foetal liver (18). Some authors did not find an effect of oestrogen on the EPO mRNA level in the kidney or in the serum level in normoxic conditions (18,19). On the other hand, in hypoxic conditions, the reports are contradictory with some reporting a suppressive effect of oestrogen on hypoxia-induced elevation of plasma EPO (19), whereas others reported that oestrogen potentiated the hypoxia-induced elevation of serum EPO (20).

In view of the varying results in the literature, it is possible that the difference observed between male and female ALS patients in the present work could be explained by differences in oestrogen levels.

This study show that the homeostatic mechanism of EPO stimulation on hypoxia is preserved in ALS; the implication of this activation for motor neuron protection merits further investigation.

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References


