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Increased Red Blood Cell Polyamines in ALS and Parkinson's Disease

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The polyamines spermidine (SPD) and spermine (SPM) are implicated in nerve cell degeneration and regeneration. Over 70% of circulating polyamines are associated with red blood cells (RBC). Against this background we have analysed RBC polyamines in two neurodegenerative disorders, amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD). Twenty patients with the sporadic form of ALS, 20 patients with PD, and 20 healthy controls were studied. The highest levels of SPD and SPM were found in the PD group where the mean values were 134 and 115%, respectively, above those of the controls. The patients with PD also presented the lowest levels of the SPD precursor, putrescine (PUTR). In the patients suffering from ALS the SPD and SPM mean levels were increased by 46 and 112%, respectively. The RBC SPD/SPM ratio in the patients suffering from PD was significantly elevated in comparison with that of ALS patient group, suggesting a different involvement of the polyamine system in these disorders. It is at present unknown if raised polyamine levels may contribute to induce the degeneration of susceptible neurons or if the increase represents a compensatory protective reaction, or simply an unspecific epiphenomenon.

Key Words: **ALS; PD; red blood cell; polyamines.**

INTRODUCTION

The polyamines spermidine (SPD) and spermine (SPM) are present in most tissues and body fluids (39). Putrescine (PUTR) and decarboxylated *S*-adenosylmethionine are the substrates for the synthesis of SPD, which is a precursor of SPM. SPD and SPM are aliphatic polycationic amines of low molecular weight and have marked basic properties. In the central nervous system they have been localized in neurons and glial cells. High concentrations of SPD are found in white matter regions, especially the spinal cord, whereas the highest concentration of SPM is found in the cerebellar cortex. PUTR levels are highest in the occipital and cerebral cortices (8, 27). Polyamines are known to be involved in the synthesis of nucleic acids and proteins during cell growth, cell differentiation and regeneration, regulation of neuronal ion channels and modulation of the NMDA (*N*-methyl-D-aspartate) subtype of glutamate receptors (22, 33, 43). Because of their activating effect on the NMDA receptor, polyamines have received considerable attention during recent years in the field of neurodegeneration. However, as regulators of nucleic acids and protein synthesis, they have also been implicated in restorative processes (4, 16). Indeed, recently, novel polyamine derivatives were found to be of some benefit as neuroprotective agents in experimental models of neurotrauma (17). Several studies have shown that intracellular levels of polyamines increase markedly in certain conditions where excitotoxicity is involved such as traumatic injuries of the brain and spinal cord and cerebral ischemia (19, 25). There is evidence to suggest that this polyamine response may be part of the events of neuronal apoptosis (18). Analysis in human body fluids have revealed that polyamines are found in blood and urine. However, detailed knowledge regarding tissue exchange and distribution of polyamines is still sparse. More than 90% of circulating SPD and over 70% of SPM are associated with the red blood cells (RBC), mainly localized intracellularly in a free form (9). Other blood components such as leukocytes and lymphocytes may also contribute to the polyamine transport (40, 41). The polyamine uptake in the RBC is energy dependent (28). Clinical studies have shown increased urinary polyamine excretion as well as elevated levels of PUTR, SPD, and SPM in RBC and muscle biopsies of a diverse group of patients with muscle cell degeneration and neuromuscular diseases in such a way that polyamine analysis was considered of interest in monitoring disease activity (23, 35, 38). Up to now, RBC polyamines have not been investigated in amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) and the above mentioned background aroused our interest in undertaking the present investigation.

MATERIALS AND METHODS

Patients and Healthy Control Subjects

Twenty patients with the sporadic form of ALS, 20 with PD, and 20 healthy control subjects were included

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TABLE 1

Main Characteristics of Patients and Control Subjects

Sex/No	Age	Disease duration (years)	Onset L/B	Norris score	Hoehn & Yahr score (1)	(2)	(3)	(4)	(5)
			ALS cases (n = 20)						
Male/11	61.0 ± 3.4*	3.3 ± 1.0	7/4	50 ± 29				—	
Female/9	63.4 ± 3.6*	4.0 ± 1.9	6/3	56 ± 23				—	
			Parkinson's cases (n = 20)						
Male/12	60.1 ± 3.9	15.2 ± 2.1	—	—	(0)	(0)	(6)	(4)	(2)
Female/8	60.0 ± 2.4	16.4 ± 4.0	—	—	(1)	(2)	(1)	(1)	(3)
			Control cases (n = 20)						
Male/10	50.5 ± 1.1	—	—	—				—	
Female/10	51.9 ± 1.0	—	—	—				—	

Note. The data are the mean ± SEM. L (Limb); B (Bulbar); *significantly older ($P < 0.05$) in comparison with the respective control.

in the study. The ALS patients were diagnosed according to El Escorial criteria (6, for an updated version see http://www.wfnals.org/Articles/elescorial1998.htm), and clinically rated according to the Norris scoring system (31). The patients with PD were diagnosed according to the UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria (21) and rated according to the Hoehn & Yahr score (20). The main characteristics of the patients and control subjects are summarized in Table 1. Five ALS patients were under medication with riluzole, an antiglutamatergic drug (24). All patients with PD were on their ordinary medication with levodopa in combination with a peripheral decarboxylase inhibitor, carbidopa, and individualized combinations of selegiline and dopamine agonists. Blood samples were collected 12 h after the last dose of levodopa. To investigate the influence of levodopa on RBC polyamines, blood samples of 5 of 20 patients were taken 12 h after the last dose and 2 h after the first morning dose. All patients had medical assistance from the Department of Neurology at the University Hospital, Uppsala, Sweden. Blood samples from healthy blood donors were obtained from the same Hospital, Department of Clinical Immunology and Transfusion Medicine. Normal health status of the controls was previously established by clinical, biochemical, and hematological screenings. All patients had haematocrit values within the normal range (men 38–49%, women 34–44%).

Sample Preparation

Venous blood (5.0 ml) was drawn into a vacutainer tube containing sodium citrate (3.1%) as anticoagulant. Blood samples were coded to conduct blind assays. After removal of the plasma the RBC pellet was washed three times with 3 vol of 0.14 M NaCl and centrifuged at 2,400g for 20 min at 4°C. Samples were ressuspended in an equal volume of isotonic saline and the RBC counts were performed by a Coulter counter (Coulter Z1, Coulter Electronics Ltd). After lysation by the addition of water 1/3 (vol/vol) on an ice bath for 30 min, the hemolysate was centrifuged at 11,220g for 20 min at 4°C. The protein free hemolysate was obtained by filtration of a 250 μl aliquot through an ultrafiltration membrane (Amicon Centrifree Micropartition System, 30,000 MW cut-off) by centrifugation in a fixed angle rotor at 2,000g for 30 min. Before ultrafiltration, 5.0 μl of 6.0 nM 1,8-diamineoctane (internal standard) was added to the hemolysate. The ultrafiltrate was immediately used for the polyamine assay.

Determination of Polyamines in RBC

Polyamines were analyzed by pre-column derivatization with 9-fluorenylmethyl chloroformate (FMOC) and separation by HPLC with fluorescence detection. The method was a modification of the procedures previously described (5, 11). Samples were derivatized by the addition of 80 μl FMOC (0.01 M in acetone) to a glass tube containing 20 μl of RBC ultrafiltrate and 60 μl 0.1 M borate buffer pH 9.0. Derivatization was allowed to proceed for 45 s and after that the excess FMOC was reacted with 100 μl 40 mM glycine. Glycine solution was prepared by dissolving it in 0.1 M borate buffer pH 9.0:acetone 50/50 (v/v) made fresh. After 45 s, the sample was diluted to 400 μl with 0.05 M sodium acetate buffer pH 2.0:acetonitrile, 30/70 (v/v). A 20 μl aliquot was injected into the HPLC system. The chromatograph consisted of a quaternary solvent delivery pump (Constametric 4100-TSP) equipped with an injector, and a column (ODS 2; Grom-Sil FMOC-Polyamine-1; 200 × 4 mm; 5 μm) maintained at 40°C. A Fluorometer (Ultrafluor Scanning Fluorescence De-

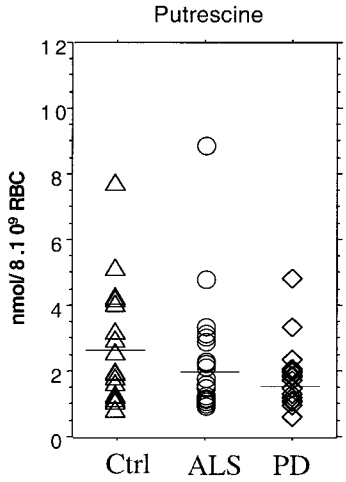

FIG. 1. Putrescine levels in RBC from 20 control subjects, 20 patients with ALS, and 20 with PD. The bars are the mean levels.

tector, Linear) was used to monitor the elution of PUTR, SPD and SPM derivatives. The excitation and emission wavelengths were set at 264 and 310 nm, respectively. The separation was carried out by gradient elution at a flow rate of 1.3 ml/min. The gradient was: 0–5 min 50% A (consisting of 800 ml sodium acetate buffer 0.05 M, pH 4.2, plus 200 ml acetonitrile)/ 50% B (consisting of 50 ml sodium acetate buffer 0.05 M, pH 4.2, plus 950 ml acetonitrile), 20 min 100% B, 20–25 min 100% B, 25–30 min 50% A/50% B. Polyamines were quantified by peak area measurements. Calibration curves were constructed with standard solutions and the polyamine levels were expressed in nmol/8.10^9 RBC (13, 29, 30).

Statistical Analysis

Values are given as Means ± 1 SD for $N = 20$ and Means ± SEM for $N < 20$. Comparisons between the groups were conducted with ANOVA and Student's t test both for unpaired and paired samples (t test). A post hoc comparison for ANOVA was done with Fisher's PLSD test (Fisher's protected least squares difference test). The relationship between two parameters was analyzed by simple regression analysis.

RESULTS

Figure 1 shows the levels of PUTR in RBC from control subjects (2.58 ± 1.72) and patients with ALS and PD. In the ALS group PUTR was within normal limits in comparison with the control group, while a 38% decrease of the mean control value was observed in RBC from patients with PD (1.55 ± 0.92).

Levels of SPD and SPM in RBC from control subjects (5.0 ± 2.0 and 4.8 ± 2.8 respectively) were within the range reported as normal in previous publications (13, 29, 30). As shown in Fig. 2, the mean value of SPD was significantly increased by 46% in the group of patients with ALS (7.3 ± 2.0) and by 134% in the RBC of patients with PD (11.8 ± 5.0). Concerning SPM, the levels increased over the mean control value (4.8 ± 2.8) by approximately 114% in the group of patients with ALS (10.3 ± 4.5) and PD disease (10.4 ± 4.7). One out of the 20 patients with PD presented extremely high RBC concentrations of SPD and SPM, 30.70 and 25.70 nmol/8.10^9 RBC as compared to the mean values of SPD and SPM excluding this patient. However, statistical analysis excluding this case in the PD group still showed significantly higher mean values of these polyamines as compared to controls.

The SPD/SPM ratios in RBC from controls, patients with ALS and PD were 0.94 ± 0.53, 0.75 ± 0.29, and 1.20 ± 0.34, respectively. There was a significant increase in the ratio in the group of patients with PD compared to ALS ($P < 0.005$).

There was no relationship between levels of PUTR, SPD, SPM and age and sex in any of the groups. Neither was there any correlation between levels of polyamines and onset type of ALS (limb or bulbar), duration of the symptoms, Norris score or treatment with riluzole. In the group of patients with PD no correlation was found between levels of polyamines and duration of the disease or Hoehn & Yahr score.

Since all patients with PD were on medication with L-dopa and carbidopa, the levels of polyamines were analyzed in RBC samples taken from 5 patients, 12 h after last dose and 2 h after the first morning dose. As shown in Table 2, no statistical significant differences were found.

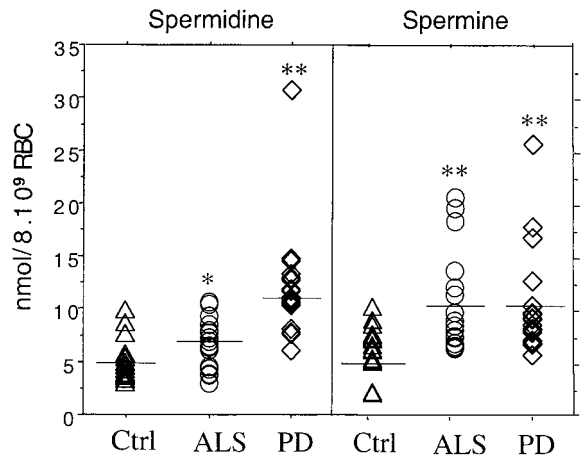

FIG. 2. Spermidine and Spermine levels in RBC from 20 control subjects, 20 patients with ALS, and 20 with PD. The bars are the mean levels. For SPD, One-way ANOVA indicated a significant difference across the three groups ($F_{2, 57} = 21.09$; $P < 0.0001$). Fishers test identified significant differences between control and ALS, $P < 0.05$*; and control and PD, $P < 0.0001$**. For SPM, $F_{2, 57} = 11.90$; $P < 0.001$, Fishers test $P < 0.0001$ for both ALS and PD versus control.

TABLE 2

Polyamine Levels in Erythrocytes from Patients with Parkinson's Disease at Different Intervals after the Administration of L-DOPA

Condition	Putr	SPD	SPM	SPD/SPM
12 h after L-DOPA	2.21 ± 0.39	9.55 ± 0.94	10.18 ± 2.63	1.09 ± 0.18
2 h after L-DOPA	2.49 ± 0.90	9.61 ± 1.01	13.49 ± 1.67	0.73 ± 0.06

Note. The data are the mean ± SEM. There was no statistically significant difference in polyamine levels.

DISCUSSION

To our knowledge, this is the first study on polyamine levels in RBC of patients with ALS and PD. It is apparent that a biochemical alteration in polyamine metabolism in these neurodegenerative disorders can be detected in the RBC. The alterations are most striking for SPD and SPM in PD. Furthermore this group also presented the lowest RBC levels of putrescine. Biosynthesis, degradation, uptake and excretion strictly regulate the polyamine content of cells. There has been increasing evidence in recent years that glutamate excitotoxicity contributes to neurodegeneration in ALS (10, 26, 36) and excitotoxic mechanisms have been proposed to be of importance also in the pathogenesis of PD (2, 10, 15, 26). In the excitotoxic model, an excess of glutamate, absolute or relative, initiates a cascade of cellular events that leads to cell death. It has been shown in an *in vitro* neuronal system, that micromolar spermine concentrations activate the NMDA receptor enhancing the glutamate-induced neurotoxicity (37). However, in a millimolar concentration spermine is reported to have a neuroprotective effect against anoxia in hippocampal slices (14). The protective effect against anoxia is interesting, as it has recently been shown that chronic vascular insufficiency lead to selective degeneration of motor neurons in mice (32).

It is known that RBC do not have the elements to synthesize polyamines and have been considered to be passive carriers from sites of polyamine release to sites of conjugation, catabolism, excretion and reuptake (40, 41). Thus, it can be speculated that the marked increases of SPD and SPM observed in RBC from patients with ALS and PD reflect increased production or decreased degradation of these polyamines in target cells affected by the degenerative process with enhancement of efflux into the extracellular space. All patients with PD were treated with L-DOPA and it is well known that the metabolism of L-DOPA and dopamine as well as the synthesis of SPD and SPM are dependent of *S*-Adenosylmethionine (SAM) (1, 3). There have been several reports on a possible influence of dopaminergic system in SAM methylation process and polyamine metabolism. Previously we reported on reduced levels of whole blood SAM and increased L-methionine *S*-adenosyl transferase activity in erythrocytes of patients with PD treated with L-DOPA (7). Experiments in rats showed a dopaminergic regulation of the key enzymes involved in polyamine biosynthesis, *S*-adenosylmethionine decarboxylase and ornithine decarboxylase (12, 34, 44). Also, it was speculated that MAO-B inhibitors interfere with polyamine metabolism (45). Thus it can not be excluded that dopaminergic drug treatment may influence polyamine levels. However, we found no difference in red blood cells polyamines 12 and 2 h after L-DOPA administration. It is interesting that the relation between the concentrations of the individual polyamines in RBC is about the same as that recently reported for the human brain by Vivó *et al.* (42). In their study, however, no difference between PD and controls was observed. Although information on polyamine transport and metabolism is sparse our findings might indicate a general involvement of the polyamine metabolism in neurodegenerative disorders. The fact that the SPD/SPM ratio of RBC from patients with PD is significantly elevated in comparison with that in ALS suggests a different defect of the polyamine system in these disorders. It is at present unknown if raised polyamine levels may contribute to induce degeneration of susceptible neurons through an excitotoxic process, or if the increase represents a compensatory protective reaction or simply an unspecific epiphenomenon. The present observations warrant further studies on polyamine metabolism in different tissues in states of neurodegeneration.

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