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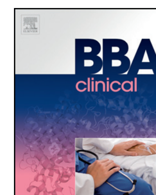

Metabolic response to glatiramer acetate therapy in multiple sclerosis patients

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ABSTRACT

Glatiramer acetate (GA; Copaxone) is a random copolymer of glutamic acid, lysine, alanine, and tyrosine used for the treatment of patients with multiple sclerosis (MS). Its mechanism of action has not been already fully elucidated, but it seems that GA has an immune-modulatory effect and neuro-protective properties. Lymphocyte mitochondrial dysfunction underlines the onset of several autoimmune disorders. In MS first diagnosis patients, $CD4^+$, the main T cell subset involved in the pathogenesis of MS, undergo a metabolic reprogramming that consist in the up-regulation of glycolysis and in the down-regulation of oxidative phosphorylation. Currently, no works exist about $CD4^+$ T cell metabolism in response to GA treatment. In order to provide novel insight into the potential use of GA in MS treatment, blood samples were collected from 20 healthy controls (HCs) and from 20 RR MS patients prior and every 6 months during the 12 months of GA administration. GA treated patients' $CD4^+$ T cells were compared with those from HCs analysing their mitochondrial activity through polarographic and enzymatic methods in association with their antioxidant status, through the analysis of SOD, GPx and CAT activities. Altogether, our findings suggest that GA is able to reduce $CD4^+$ T lymphocytes' dysfunctions by increasing mitochondrial activity and their response to oxidative stress.

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1. Introduction

Multiple sclerosis (MS) is a primary inflammatory demyelinating autoimmune disorder of the central nervous system (CNS) affecting mainly young people aged between 20 and 40 years at disease onset. In early stages, the disease is characterized by infiltration and activation of T cells and accumulation of monocyte-derived macrophages, which promote destruction of the myelin sheath leading to the formation of focal demyelinated lesions [1]. The disease involves a life-long, unpredictable course generally categorized as relapsing-remitting, secondary progressive, and primary progressive, though all these courses entail a progressive destruction of myelin [2]. Previous studies demonstrated that MS induces alteration in energy metabolism and in oxidants/antioxidants balance that can be monitored in serum of MS patients [3]. Moreover, recent evidence suggests that mitochondrial dysfunctions contribute to neurological disorders [4,5], supporting the role of mitochondria as a potential therapeutic target in MS [6]. Interestingly, also $CD4^+$ T lymphocytes, the main T cell population involved in the pathogenesis of MS, present metabolic alterations. This is an intriguing aspect, because these cells could be used as bio-energetic markers. In particular, previous studies carried out by our group demonstrated that $CD4^+$ T cells of MS patients have a reduction in oxygen consumption in association with an increase in the activity of glycolytic enzymes respect to the cells of control ones [7]. These findings lead us to propose $CD4^+$ T cell bio-energetic status as a marker for diagnosis and *follow up* for MS [7]. During the last decade, new therapies have been shown to significantly improve MS disease course. Currently, 13 different drugs with ten different active components are licensed in the European Union [EU] and the United States [US] for the treatment of MS. These drugs can be categorized into first-, second- and third-line treatment. Among these, Glatiramer acetate [GA; commercial name Copaxone] is a first-line immunomodulatory therapy [8] and a widely used disease-modifying drug indicated for the reduction of relapses in patients with relapsing-remitting MS (RRMS). GA is a synthetic compound of the four amino acids (Glu, Ala, Lys, Tyr) that are most common in myelin basic protein [9]. Even if its mechanism of action has not been already fully elucidated, it seems that GA has an immunomodulatory effect and neuroprotective properties [10,11,12]. Moreover, GA skews CD4 T cells differentiation from pathological Th1

Abbreviations: MS, Multiple Sclerosis; RRMS, Relapsing-Remitting Multiple Sclerosis; ROS, reactive oxygen species; CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; Th, T helper; OXPHOS, oxidative phosphorylation; PBMC, peripheral blood mononuclear cell; RCR, respiratory control ratio; GR, glutathione reductase; CS, citrate synthase; SOD, superoxide dismutase; CAT, catalase; GPX, glutathione peroxidase; HK, hexokinase; PFK, phosphofructokinase; MCT, mono-carboxylate transporters.

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toward regulatory Th2 phenotypes secreting IL-4 and 10 [13] and affects innate immune cells including macrophages and dendritic cells [14,15]. GA probably also increases the frequency of FoxP3-expressing regulatory T cells, effects that are at least partly mediated by the generation of anti-inflammatory antigen-presenting cells, allowing the differentiation of naïve T cells into Th2 or Th3 and regulatory T cells [16,17]. Particularly, it was shown that GA influence monocyte/macrophage polarization by shifting the balance from pathological M1 toward the M2 regulatory phenotypes [15]. The majority of studies focusing on the mechanism of action of GA were conducted *in vitro* and no information are available about GA effect on $CD4^+$ T cell metabolism. Ruggieri et al. demonstrated that GA is able to restore a correct balance in the process of apoptosis of cultured PBMCs from MS patients [18]. In order to provide more insight into the effect of GA treatment on $CD4^+$ T cell metabolism, in the present study we investigated the metabolic characteristics of this cell subset in association with response to oxidative stress in GA treated MS patients in a 12 months follow up study.

2. Materials and methods

2.1. Ethical permission

The study was approved by the Vito Fazzi Hospital Ethics Committee (Lecce, Italy) and informed consent was obtained from each patient prior to entry into the study, according to the declaration of Helsinki.

2.2. Participants/study population

20 patients diagnosed with RRMS in Vito Fazzi Hospital with an age range of 19–45 years were included into the study. Patients had to be without any immune-modulatory treatment at least 6 months prior to study entry. For each patient, blood samples were obtained at baseline (untreated) and every 6 months during GA (Copaxone® – Teva) treatment (20 mg s.c./day) for a period of 12 months. Written informed consent was obtained from each individual before the start of the study. Blood samples were also collected from 20 sex and age matched healthy controls (HCs).

2.3. $CD4^+$ T cell isolation

PBMC fractions were isolated from whole blood using Ficoll-Paque density-gradient centrifugation. $CD4^+$ T cells were purified by negative selection using an indirect magnetic cell sorting kit (MiltenyiBiotec, Bergisch Gladbach, Germany). In summary, human $CD4^+$ T cells were isolated by depletion of non-$CD4^+$ T cells. Non-$CD4^+$ T cells were indirectly magnetically labelled with a cocktail of biotin-conjugated monoclonal antibodies, as primary labelling reagent, and *anti*-biotin monoclonal antibodies conjugated to MicroBeads, as secondary labelling reagent. The magnetically labelled non-$CD4^+$ T cells were depleted by retaining them on MACS® Column in the magnetic field of a MACS Separator, while the unlabeled cells passed through the column.

2.4. Polarographic measurement of respiratory rate

$1 \cdot 10^6$ cells $CD4^+$ T cells were suspended in buffer containing 10 mM HEPES, 143 mM NaCl, 4 mM KCl, pH 7.4. Oxygen consumption was measured at 36 °C by a Clark-type oxygen probe (Oxygraph, Hansatech Instruments, King's Lynn, UK), in the presence of mitochondrial respiratory substrates (5 mM pyruvate and 5 mM malate) and 0.5 mM ADP. The rate of oxygen uptake (V) by $CD4^+$ T cell mitochondria was expressed as nmol $O_2 \cdot ml^{-1} \cdot minute^{-1}$. The respiratory control ratio (RCR) was calculated by dividing V_3(rate of oxygen uptake measured in the presence of respiratory substrates + ADP) by V_4(rate of oxygen uptake measured with respiratory substrates alone).

2.5. ΔΨ measurement

$CD4^+$ T cells were incubated with 2 μM JC-1 (Molecular Probes, Eugene,*CA*) for 20 min and then fluorescence of J-aggregates and J-monomers was measured using excitation/emission wavelengths of 535/595 nm and 485/535 nm, respectively.

2.6. Respiratory complexes activity

$CD4^+$ T lymphocytes were resuspended in Mito buffer (2 mM HEPES, 0.1 mM EGTA, 250 mM sucrose, pH 7.4) supplemented with a protease inhibitor mixture, subjected to three freeze-thawing cycles and, after the addition of 10 mM triethanolamine and 0.1 mg/ml digitonin, were incubated for 10 min on ice, homogenised and centrifuged at 1000 ×*g* for 10 min at 4 °C. The supernatant was saved and the pellet resuspended in the same volume of Mito buffer supplemented with 0.1 mg/ml digitonin, homogenised and centrifuged once again. Supernatants were mixed and centrifuged at 12,000 ×*g* for 15 min at 4 °C, and the mitochondria-rich pellet was resuspended in Mito buffer. A total of 10–40 μg of proteins were used to determine the activity of each complex. The assays were performed at 37 °C (except for citrate synthase activity that was assayed at 30 °C) using microcuvettes (volume 100 μl).

Activities of complex I, II and III were determined according to the method described by Frazier and Thorburn [19]. Complex IV activity was determined using a COX assay kit (CYTOCOX1) from Sigma-Aldrich. All activities were expressed as mU/mg of proteins.

Citrate synthase [CS], a ubiquitous mitochondrial matrix enzyme, serving as a mitochondrial marker, was measured in the presence of acetyl-CoA and oxaloacetate by monitoring the liberation of CoA-SH coupled to dithiobis(2-nitrobenzoic) acid spectrophotometrically at 412 nm. Protein concentration was determined by the Bradford method and calculated according to bovine serum albumin (BSA) standard curve.

2.7. Antioxidant enzyme activities

The superoxide dismutase (SOD) activity was measured using the Sigma SOD assay kit-WST (Sigma-Aldrich, Switzerland) following the manufacturer's instructions.

Catalase (CAT) activity was assayed by the method of Luck [20]. The assay mixture consisted of H_2O_2-phosphate buffer (12.5 mM H_2O_2 in 0.067 M phosphate buffer, pH 7.0) and cell homogenate. Absorbance changes were recorded at 240 nm for 3 min. Results were expressed as U/mg protein using molar extinction coefficient of H_2O_2($71 \times M^{-1}\ cm^{-1}$).

Glutathione peroxidase (GPx) activity was determined according to Lawrence and Burk [21] with modifications. The activity was measured as a decrease in absorbance at 340 nm for 5 min at 25 °C based on a coupled reaction with glutathione reductase (GR) in the presence of GSH and H_2O_2. Cell homogenate was added to each well containing 100 μL of 25 mM KH_2PO_4, 0.5 mM EDTA pH 7.4, 0.5 mM $NaNO_3$, 0.3 mM NADPH, 0.64 units of GR, and 1 mM GSH. The reaction was initiated by adding 0.1 mM H_2O_2. Values were corrected for nonenzymatic oxidation of GSH and NADPH by H_2O_2. GPx activity was expressed as U/mg of protein using 6.22 $mM^{-1}\ cm^{-1}$ as the extinction coefficient.

2.8. Hexokinase and phosphofructokinase activity assays

A total of 30–50 μg of cellular proteins were used. The assays were performed at 37 °C using microcuvettes(volume 100 μl). Hexokinase activity was quantified following the method described by Tielens et al. [22]. Briefly, 5 μl of sample dissolved in 35 μl of water was added to a mix containing 40 mM Tris, 22 mM Mg-acetate, 10 mM β mercaptoethanol, $NADP^+$ 0.75 mM, 1 U/ml G6PDH, 10 mM ATP. After

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