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Biomolecules damage and redox status abnormalities in Fabry patients before and during enzyme replacement therapy

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ABSTRACT

Fabry disease (FD) is caused by deficient activity of the lysosomal enzyme α-galactosidase A. Its substrates, mainly globotriaosylceramide (Gb3), accumulate and seem to induce other pathophysiological findings of FD. Once enzyme replacement therapy (ERT) is not completely efficient on preventing disease progress in FD patients, elucidating the underlying mechanisms in FD pathophysiology is essential to the development of additional therapeutic strategies. We investigated 58 Fabry patients (23 male and 35 female) subdivided into two groups (at diagnosis and during long-term ERT) and compared them to healthy individuals. Fabry patients at diagnosis presented altered glutathione (GSH) metabolism (higher GSH levels, lower glutathione peroxidase – GPx – and normal glutathione reductase – GR - activities), higher lipid peroxidation levels (thiobarbituric acid reactive species - TBARS - and malondialdehyde - MDA), nitric oxide ($NO^{\cdot}$) equivalents and urinary Gb3. Fabry patients on ERT presented GSH metabolism similar to controls, although lipid peroxidation and urinary levels of $NO^{\cdot}$ equivalents remained higher whereas Gb3 levels were lower than at diagnosis but still higher than controls. These data demonstrated that redox impairment occurs in Fabry patients before and after ERT, probably as a consequence of Gb3 accumulation, providing targets to future therapy approaches using antioxidants in combination with ERT in FD.

1. Introduction

Fabry disease (FD, OMIM 301500) is a lysosomal disorder caused by mutations on α-galactosidase A (α-gal A, EC 3.2.1.22) coding gene (GLA), with systemic deposition of its substrates (glycosphingolipids with terminal α-galactosyl, mainly globotriaosylceramide – Gb3). The estimated incidence of classical FD is about one in 40,000 males [1], but non-classical phenotypes are much more frequent [2,3].

Differently from how it seems to be in the early FD descriptions [4,5], studies in last decades have shown a multi-organ complex disease [1,6–8]. Beyond direct accumulation of substrates, other mechanisms are now known to play an important role in FD pathophysiology, such as inflammation, oxidative stress and DNA damage [1,7,9–12]. Moreover, Gb3 was capable to induce proinflammatory profile in cultured peripheral blood mononuclear cells [11] and increased intracellular reactive oxygen species (ROS) in cultured vascular endothelial cells [10].

Also, major complications in FD – cerebrovascular, renal and cardiac disease - are due to vasculopathy that seems to involve cascade events in adhesion molecules, cytokines and pro-inflammatory effect on leucocytes, endothelial cells and vascular smooth muscle cells [1,6]. Studies in an animal model of FD [13] and in dermal and cerebral vessels of Fabry patients [7] are in line with the hypothesis that nitric oxide and nitrosative stress may be key elements in FD vasculopathy.

Oxidative stress has been pointed as an important element in physiological and pathological processes, including inflammatory ones [14] as well as other lysosomal disorders [15–17]. Our research has been focusing in the mechanisms by which oxidative stress occurs in Fabry disease and demonstrated in Fabry patients under ERT (for about 24 months) interesting data supporting the idea of oxidative and inflammatory imbalance [9], although a deep investigation in patients

Abbreviations: α-gal A, α-galactosidase A; Cr, creatinine; ERT, enzyme replacement therapy; FD, Fabry disease; GCL, glutamate cysteine ligase; Gb3, globotriaosylceramide; GLA, α-galactosidase A coding gene; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; Lyso-Gb3, globotriaosylsphingosine; MDA, malondialdehyde; NO_3^-, nitrate; $NO^{\cdot}$, nitric oxide; NO_2^-, nitrite; $ONOO^-$, peroxinytrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive species.

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before treatment remains necessary. Therefore, in the present study we investigated in Fabry patients, before and during long-term ERT, the following parameters: the metabolism of the main intracellular antioxidant system (glutathione), the oxidative damage to lipids and proteins, the nitrosative stress and the levels of the major accumulated substrate in FD (Gb3).

2. Material and methods

2.1. Subjects

The study was performed in 23 male and 35 female Fabry patients (all of them with the classical phenotype), with results compared to the obtained in 48 healthy individuals. Subjects were divided into three groups: 1) 12 Fabry patients (median age 34.5 years; range 19–64) diagnosed but not receiving ERT yet – called "Fabry" group; 2) 11 Fabry patients under ERT treatment (agalsidase alfa — Replagal®0.2 mg/kg or agalsidase beta — Fabrazyme® 1.0 mg/kg; median ERT time period 5.5 years) every two weeks (median age 44 years; range 24–67) – called "Fabry ERT" group; 3) 10 healthy individuals matched by age and sex (median age 33.5 years; range 20–67) – called "Control" group. Some patients collected only urine samples, then medians and number of subjects in each group was bigger for urine biochemical determinations: Fabry group (n = 17; median age 27 years; range 4–64); Fabry ERT group (n = 22; median age 39.5 years; range 20–67; median ERT time period 2.1 years); Control group (n = 38; median age 45 years; range 14–67).

Male patients' diagnosis was performed by identification of deficient α-gal A activity in plasma and confirmed in leucocytes. For the females, diagnosis was confirmed by molecular analysis of the GLA gene and identification of a pathogenic mutation.

The study was approved by the *Hospital de Clínicas de Porto Alegre* Ethics Committee (number 100177). Informed consent was obtained from all the participants.

2.2. Samples

Heparinized blood and urine samples were collected from patients and controls concomitantly. Whole blood was separated by centrifugation (1000 × *g* for 10 min), plasma removed by aspiration, aliquoted and frozen at −80 °C until analysis. Cells were washed three times with cold saline solution and then erythrocyte lysates prepared by addition of 1 mL distilled water to 100 μL erythrocytes aliquot. Lysate aliquots were maintained at −80 °C until analysis. Urine samples were collected in sterile flasks, homogenized, deposited on 10–10 cm virgin filter papers (Whatman 903) to Gb3 quantification and the remaining volume aliquoted and frozen at −80 °C until nitrate and creatinine determinations.

2.3. Biochemical determinations

2.3.1. Erythrocyte glutathione (GSH) metabolism parameters

2.3.1.1. Reduced glutathione (GSH). GSH erythrocyte levels were measured as described by Browne and Armstrong [18] adapted to microplate. The resulting sample fluorescence was measured (λexcitation = 350 nm, λemission = 420 nm) and compared to calibration curve prepared with GSH solutions. Results were expressed as nmol/mg protein.

2.3.1.2. Glutathione peroxidase (GPx). Erythrocyte GPx activity was measured by using RANSEL® kit (Randox lab), that is based on GPx catalysis of GSH oxidation to GSSG (oxidized GSH) and subsequent reduction to GSH in the presence of glutathione reductase (GR) and NADPH concomitantly oxidated to NADP. Absorbance decrease after 1 and 2 min at 340 nm was measured and results were expressed as mU/mg protein.

2.3.1.3. Glutathione reductase (GR). Erythrocyte GR activity was determined as described by Carlberg and Mannervik [19] in which GR catalyses GSSG reduction to GSH with NADPH oxidation to NADP. Absorbance was monitored at 340 nm and results were expressed as U/mg protein.

2.3.2. Lipid peroxidation parameters

2.3.2.1. Thiobarbituric acid reactive species (TBARS) plasma levels. Lipid peroxidation was firstly estimated by measuring TBARS according to the method described by Ohkawa, Ohishi and Yagi [20] in which samples are heated with TBA at low pH and a pink chromogen is read at 535 nm. Results were expressed as nmol/mg protein.

2.3.2.2. Malondialdehyde (MDA) plasma levels. To confirm the results found by TBARS technique, we also measured lipid peroxidation by the high performance liquid chromatography (HPLC) method described by Esterbauer and Cheeseman [21] with slight modifications. This method is more specific than TBARS, once it detects only the lipid peroxidation end product MDA. Briefly, to a plasma aliquot were added 28% trichloroacetic acid and distilled water. After centrifugation, supernatant MDA was removed and measured by HPLC using an amino-phase column with 30 mM acetonitrile, pH 7.4 tris buffer (1:9, v/v). Effluent was monitored at 267 nm and calibration curve was done with MDA standard solutions. Results were expressed as log of mM MDA.

2.3.3. Thiol (SH) groups plasma levels

Total plasma thiol content, an indirect method to determine oxidative damage to proteins, was measured as described by Aksenov and Markesbery [22]. The method is based on reduction of 5,5,-dithiobis(2-nitrobenzoic acid) (DTNB) into a yellow product (TNB) by sample thiols. TNB absorption was measured spectrophotometrically at 412 nm. Results were expressed as nmol TNB/mg protein.

2.3.4. Nitric oxide (NO•) urinary levels

NO• is measured in biological fluids indirectly by quantifying the NO• stable products nitrate and nitrite. It was used a commercial nitrate/nitrite kit (Cayman Chemical, Ann Arbor, MI) that is based on nitrate conversion to nitrite by nitrate reductase and subsequent Griess reaction with nitrite leading to an azo product formation which absorption was read in 540 nm. Results were expressed as μmol/mg creatinine.

2.3.5. Urinary globotriaosylceramide (Gb3)

A 5-cm diameter filter paper disc was punched from each sample and processed as described by C. Auray-Blais et al. [23] with some modifications. The internal standard (IS) $C_{17:0}$-Gb3 was added to the urine samples before injection into the LC-MS/MS (liquid chromatography-tandem mass spectrometry) system. For LC, it was used an Alliance 2695 system with stepwise gradient elution with mobile phases A (ammonium acetate 2 mM + 0.1% formic acid in water) and B (ammonium acetate 2 mM + 0.1% formic acid in methanol). A Discovery® C8, 5 μm column (577508-U) was used for separation, at 45 °C and the total analysis run time was 4.5 min. MS/MS was carried out using a Quattro micro tandem quadrupole instrument (Waters Micromass, Manchester, UK) with electrospray ionization operated in positive ion mode. The multiple reaction monitoring mode was used for the measurement of IS and total Gb3 isoforms. Results were expressed as μg/mg creatinine.

2.3.6. Protein quantification

Plasma and erythrocyte protein levels were determined respectively, by Biuret method – using Labtest® kit (Labtest Diagnóstica, Lagoa Santa, MG, Brazil) and by Lowry method [24], both measured in spectrophotometer.