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Experimental evidence of oxidative stress in plasma of homocystinuric patients: A possible role for homocysteine

Camila Simioni Vanzin [a,b], Giovana Brondani Biancini [a,b], Angela Sitta [a,b], Carlos Alberto Yasin Wayhs [b,c], Izabela Netto Pereira [b], Francieli Rockenbach [c], Solange Cristina Garcia [c], Angela Terezinha de Souza Wyse [a], Ida Vanessa Doederlein Schwartz [b], Moacir Wajner [a,b], Carmen Regla Vargas [a,b,c,*]

[a] Programa de Pós-Graduação em Ciências Biológicas:Bioquímica da Universidade Federal do Rio Grande do Sul (UFRGS), Ramiro Barcelos 2700, Porto Alegre, RS, 90035-000, Brazil
[b] Serviço de Genética Médica do Hospital de Clínicas de Porto Alegre, Ramiro Barcelos 2350, Porto Alegre, RS, 90035-903, Brazil
[c] Programa de Pós-Graduação em Ciências Farmacêuticas da Universidade Federal do Rio Grande do Sul (UFRGS), Ipiranga, 2752, Porto Alegre, RS, 90610-000, Brazil

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ABSTRACT

Homocystinuria is an inherited disorder biochemically characterized by high urinary excretion of homocystine and increased levels of homocysteine (Hcy) and methionine in biological fluids. Affected patients usually have a variety of clinical and pathologic manifestations. Previous experimental data have shown a relationship between Hcy and oxidative stress, although very little was reported on this process in patients with homocystinuria. Therefore, in the present study we evaluated parameters of oxidative stress, namely carbonyl formation, malondialdehyde (MDA) levels, sulfhydryl content and total antioxidant status (TAS) in patients with homocystinuria at diagnosis and under treatment with a protein restricted diet supplemented by pyridoxine, folate, betaine, and vitamin B_{12}. We also correlated plasma Hcy and methionine concentrations with the oxidative stress parameters examined. We found a significant increase of MDA levels and carbonyl formation, as well as a reduction of sulfhydryl groups and TAS in plasma of homocystinuric patients at diagnosis relatively to healthy individuals (controls). We also verified that Hcy levels were negatively correlated with sulfhydryl content and positively with MDA levels. Furthermore, patients under treatment presented a significant reduction of the content of MDA, Hcy and methionine concentrations relatively to patients at diagnosis. Taken together, the present data indicate that lipid and protein oxidative damages are increased and the antioxidant defenses diminished in plasma of homocystinuric patients, probably due to increased reactive species elicited by Hcy. It is therefore presumed that oxidative stress participates at least in part in the pathogenesis of homocystinuria.

1. Introduction

Homocystinuria is an inherited metabolic disorder characterized by high levels of homocysteine (Hcy) in biological fluids. Cystathionine β-synthase (CBS) deficiency is the most frequently encountered cause of homocystinuria, but also genetic defects involving the enzymes methylene-H_4folate reductase (MTHFR) and methionine synthase may lead to abnormal accumulation of homocysteine in biological fluids. In contrast to most cases of CBS deficiency, hypermethioninemia is absent in the latter conditions. CBS deficiency is inherited as an autosomal recessive trait. Some patients have small residual activities of CBS, whereas in others this activity cannot be measured. Individual affected by CBS deficiency usually have a variety of clinical and pathologic abnormalities, such as ectopia lentis (dislocation of the ocular lens), osteoporosis, thinning and lengthening of the long bones, thromboembolism and mental retardation. Management of CBS deficiency has two major aims: control or elimination of biochemical abnormalities and supportive treatment of complications [1]. Treatment with the cofactor of CBS, pyridoxine (vitamin B_6), at a dose of approximately 200 mg/day should be given to those shown to be B_6-responsive. The majority of B_6-responsive individuals also require a protein-restricted diet for metabolic control. B_6-nonresponsive neonates require a methionine-restricted diet with frequent metabolic monitoring [2]. B_6-responsive individuals generally have milder, or more slowly developing manifestations than those B_6-nonresponsive [1]. In a large international survey, virtually equal proportions of patients were judged to be B_6-responsive and B_6-nonresponsive [3]. Treatment with betaine provides an alternate pathway to convert excess of homocysteine into metionine and may help to prevent complications, particularly thrombosis [4]. The dose of

Abbreviations: CBS, cystathionine β-synthase; Hcy, homocysteine; MTHFR, methylene-H_4folate reductase; ROS, reactive oxygen species; MDA, malondialdehyde; TAS, total antioxidant status; Met, methionine; PBSG, protein-bound sulfhydryl groups.

* Corresponding author at: Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Rua Ramiro Barcelos, 2350, Bairro: Bom Fim, Porto Alegre, RS, Brazil, 90035-003. Fax: +55 51 3359 8309.
E-mail address: crvargas@hcpa.ufrgs.br (C.R. Vargas).

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betaine used has been 6 to 9 g/day in two divided doses [5]. Furthermore, folate and vitamin B_{12} optimize the conversion of homocysteine to methionine by methionine synthase, thus helping to decrease the plasma homocysteine concentration. Folic acid is given orally at 5 mg/day and vitamin B_{12} is given as hydroxocobalamin at 1 mg IM per month to homocystinuric patients [2].

Although the pathogenesis of homocystinuria is not fully established, some experimental works have stressed a role for oxidative stress as a possible mechanism of tissue damage in this disorder [6]. This pathological condition can be defined as a disturbance in the prooxidant-antioxidant balance in favor of the former, leading to potential cell damage [7,8]. In this scenario, most thiols autoxidize in the presence of transition metal catalysts and molecular oxygen, and the thiol molecule homocysteine under certain conditions can similarly undergo autoxidation, generating the reactive oxygen species (ROS) superoxide anion, hydrogen peroxide, or hydroxyl radical. It has been suggested that these species may lead to endothelial cellular damage, lipid peroxidation and inhibition of nitric oxide-related cerebrovascular responses [9–12]. Some studies have investigated the role of oxidative stress in animal models of hyperhomocysteinemia [13–15], but scarce studies have been published evaluating parameters of oxidative stress in patients with homocystinuria.

Therefore, in the present work, we evaluated important parameters of oxidative stress, namely carbonyl content, sulfhydryl content, malondialdehyde (MDA) levels and total antioxidant status (TAS) in patients with homocystinuria at diagnosis and under treatment and in healthy individuals. We also correlated plasma homocysteine and methionine concentrations with the oxidative stress parameters examined.

2. Materials and methods

2.1. Patients and controls

Subjects with homocystinuria were recruited from the Medical Genetic Service of Hospital de Clínicas de Porto Alegre, Brazil. Plasma samples were obtained from 9 patients with homocystinuria at diagnosis (median age: 10 years; range: 4–27 years), 11 patients with homocystinuria under treatment (median age: 19 years; range: 12–32 years), and 11 healthy individuals with comparable age and sex (median age: 22 years; range: 5–30 years). The main features of patients with homocystinuria are summarized in Table 1. All patients were diagnosed after the neonatal period by identification of abnormal elevated concentrations of homocysteine and methionine in plasma. The major clinical manifestations were ectopia lentis, seizures, developmental delay, thinning and lengthening of the long bones (*marfanoid* appearance). The average duration of treatment was 11 years (range: 5–20 years). The treatment consisted of a protein-restricted diet supplemented by pyridoxine (median dose: 500 mg/day; range: 100–750 mg/day), folic acid (median dose: 5 mg/day; range: 2–5 mg/day), betaine (median dose: 6 g/day; range: 2–6 g/day) and vitamin B_{12} (median dose: 1 mg IM/month).

The present study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre, RS, Brazil. Informed consent was obtained according to the guidelines of our committee.

2.2. Plasma preparation

Plasma was separated from whole blood samples obtained from individuals (controls and patients with homocystinuria) by venous puncture with heparinized vials. Whole blood was centrifuged at 3000 ×*g* for 10 min at 4 °C, plasma was removed by aspiration and frozen at −80 °C until analysis.

Table 1
Clinical and metabolic features of patients with homocystinuria.

Patient	Sex	Age at diagnosis, years	Duration of treatment, years	B_6-responsive
*C.C.	M	10	–	–
*I.C.	M	10	–	
*A.C.	M	4	–	–
*R.S.	F	9	–	–
*B.S.	F	10	–	–
*J.C.	F	10	–	–
*K.S.	M	5	–	–
*D.S.	F	27	–	–
*B.S.	F	8	–	–
A.B.	M	6	6	No
L.L.	M	4	10	Yes
A.P.	M	23	5	NA
L.Z.	M	2	13	Yes
M.S.	M	6	13	No
N.C.	F	4	19	No
F.R.	F	8	11	No
A.C.	F	8	20	No
R.C.	M	13	19	No
D.C.	M	10	10	No
R.F.	M	12	6	NA

NA indicates not available.
* Patients with homocystinuria at diagnosis (untreated).

2.3. Carbonyl measurement

Carbonyl content was measured according to the method described by Levine et al. [16]. Briefly, duplicate aliquots of plasma (100 μL) were treated with 100 μL of 28% trichloroacetic acid. The tubes were centrifuged at 8000 ×*g* for 10 min to obtain the protein pellet. One mililiter of 10 mM 2.4-dinitrophenylhydrazine (DNPH) prepared in 2 M HCl or 1.0 mL of 2 M HCl (blank) was added to the precipitates and incubated at 37 °C for 90 min. After, the samples were centrifuged and the DNPH excess was removed with ethanol–ethyl acetate 1:1 (v/v). The final protein pellet was dissolved in 200 μL of 6 M guanidine hydrochloride. Quantification was performed using a spectrophotometer at 370 nm. The carbonyl content was calculated using a millimolar absorption coefficient of the hydrazone (21.000 M^{-1} cm^{-1}). Values of carbonyl content were expressed in nmol carbonyl/mg protein.

2.4. Sulfhydryl measurement

This assay is based on the reduction of 5.5′-dithio-bis (2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative whose absorption is measured spectrophotometrically at 412 nm [17]. Thirty microliters of plasma were incubated with an equal volume of DTNB at room temperature for 30 min in a dark room. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB.

2.5. Malondialdehyde (MDA) measurement

The MDA levels in plasma were measured by high-performance liquid chromatography (HPLC)-VIS, as described by Grotto et al. [18]. A volume of 75 μl of plasma was added to 25 μl of standard (dimethylacetal) or water plus 25 μl of 3 N NaOH and the mixture was incubated at 60 °C for 30 min in a shaking water bath system. After this, 125 μl of 6% H_3PO_4 and 125 μl of 0.8% TBA were added and the mixture was heated at 90 °C for 45 min. Then, the mixture was cooled, 50 μl of 10% sodium dodecyl sulfate (SDS) was added and the extraction with 300 μl of *n*-butanol was performed, followed by centrifugation. Twenty microliters of the butanol layer was injected into HPLC with a visible detector, using a reverse-phase column. The mobile phase was a mixture of Milli-Q water and methanol