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Short Communication

Homocysteine contribution to DNA damage in cystathionine β-synthase-deficient patients

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ABSTRACT

High blood levels of homocysteine (Hcy) are found in patients affected by homocystinuria, a genetic disorder caused by deficiency of cystathionine β-synthase (CBS) activity, as well as in nutritional deficiencies (vitamin B_{12} or folate) and in abnormal renal function. We previously demonstrated that lipid and protein oxidative damage is increased and the antioxidant defenses diminished in plasma of CBS-deficient patients, indicating that oxidative stress is involved in the pathophysiology of this disease. In the present work, we extended these investigations by evaluating DNA damage through the comet assay in peripheral leukocytes from CBS-deficient patients, as well as by analyzing of the in vitro effect of Hcy on DNA damage in white blood cells. We verified that DNA damage was significantly higher in the CBS-deficient patients under treatment based on a protein-restricted diet and pyridoxine, folic acid, betaine and vitamin B_{12} supplementation, when compared to controls. Furthermore, the in vitro study showed a concentration-dependent effect of Hcy inducing DNA damage. Taken together, the present data indicate that DNA damage occurs in treated CBS-deficient patients, possibly due to high Hcy levels.

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1. Introduction

Homocystinuria is an inherited error of metabolism caused by a deficiency of cystathionine β-synthase (CBS) activity. The primary metabolic consequence of this deficiency is tissue accumulation of homocysteine (Hcy), resulting in hyperhomocysteinemia and hypermethioninemia. Furthermore, homocystine, the oxidized product of Hcy, is excreted at high concentrations in urine. Dislocation of the optic lens, osteoporosis, thinning and lengthening of the long bones, mental retardation, and thromboembolism affecting large and small arteries and veins are the most common clinical features of homocystinuria (Mudd et al., 2001). Management of CBS-deficient patients includes a protein-restricted diet and the administration of pyridoxine (for those responsive to this vitamin), folic acid, betaine and vitamin B_{12} in order to control the elevated plasma Hcy concentration and to prevent the complications of homocystinuria (Mudd et al., 2001; Rao et al., 2008).

It is important to emphasize that not only deficient activity of CBS, but also other genetic defects may lead to abnormal accumulation of Hcy in the plasma, such as mutations that inactivate the enzymes methylenetetrahydrofolate reductase (MTHFR) and methionine synthase (Mudd et al., 2001). In addition to genetic disorders, other factors may lead to hyperhomocysteinemia, such as nutritional deficiencies of vitamin B_{12} or folate and abnormal renal function (Fowler, 2008). Hyperhomocysteinemia is an independent risk factor for cardiovascular disease and is associated with Alzheimer's disease and vascular dementia (Faraci and Lentz, 2004; Sánchez-Moreno et al., 2009; Seshadri et al., 2002).

Although the underlying mechanisms by which Hcy exerts its deleterious effects remain unexplained, some studies have demonstrated that DNA damage, which may occur by induction of DNA hypomethylation and generation of reactive oxygen species (ROS), seems to be induced by high levels of Hcy (Lin et al., 2007; Oikawa et al., 2003; Picerno et al., 2007). Furthermore, it has been suggested that oxidative stress, which is defined as a serious imbalance between the production of reactive species and the tissue antioxidant defenses (Halliwell and Gutteridge, 2007; Halliwell and Whiteman, 2004), plays an important role in the pathophysiology of homocystinuria (Faraci and Lentz,

Abbreviations: CAT, catalase; CBS, cystathionine β-synthase; DI, damage index; Hcy, homocysteine; LC–MS/MS, liquid chromatography electrospray tandem mass spectrometry; MTHFR, methylenetetrahydrofolate reductase; ROS, reactive oxygen species; SAH, *S*-adenosylhomocysteine; SOD, superoxide dismutase; SPSS, Statistical Package for the Social Sciences; tHcy, total homocysteine.

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2004; Perna et al., 2003). In agreement with this hypothesis, data obtained from animal models of hyperhomocysteinemia have shown an association between Hcy and oxidative stress (da Cunha et al., 2011; Matté et al., 2009; Robert et al., 2005). Moreover, we recently demonstrated that lipid and protein oxidative damage is increased and the antioxidant defenses diminished in plasma of CBS-deficient patients, probably due to increase of reactive species generation induced by Hcy (Vanzin et al., 2011).

In the present work, we extended these investigations analyzing DNA damage in white blood cells from treated CBS-deficient patients using the comet assay. We also evaluated the in vitro effect of different concentrations of Hcy on DNA damage in white blood cells.

2. Material and methods

2.1. In vivo study

Subjects with homocystinuria due to CBS deficiency and aged-matched controls were recruited from the Medical Genetic Service of Hospital de Clínicas de Porto Alegre, Brazil.

Blood samples from 9 patients with homocystinuria under treatment (mean age: 21.44 ± 7.07 years) and from 9 healthy individuals (mean age: 26.44 ± 3.71 years) were used in the experiments. The treatment of the patients consisted of a protein-restricted diet supplemented by pyridoxine (mean dose: 425.0 ± 236.1 mg/day), folic acid (mean dose: 4.40 ± 1.34 mg/day), betaine (mean dose: 6 g/day) and vitamin B_{12} (mean dose: 1 mg intramuscularly/month). The average duration of treatment was 12.33 ± 7.66 years. Total homocysteine (tHcy) mean levels in plasma of treated CBS-deficient patients were 166.5 ± 117.1 μM.

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 the committee.

2.2. In vitro study

Leukocytes isolated from normal whole blood were incubated with various concentrations of Hcy (10, 50, 100, 200 or 300 μM) at 37 °C for 6 h (Hartmann et al., 2003; Tice et al., 2000). This range of Hcy concentrations was based on the normal plasma Hcy levels (10 μM) and the concentrations found in the blood from treated and not treated homocystinuric patients (50, 100, 200 or 300 μM).

2.3. Single cell gel electrophoresis (comet assay)

The alkaline comet assay was performed as described by Singh et al. (1988) in accordance with general guidelines for the use of the comet assay (Hartmann et al., 2003; Tice et al., 2000). Isolated human leukocytes were suspended in agarose and spread into a glass microscope slide pre-coated with agarose. Agarose was allowed to set at 4 °C for 5 min. Slides were incubated in ice-cold lysis solution to remove cell proteins, leaving DNA as 'nucleoids'. After the lysis procedure, slides were placed on a horizontal electrophoresis unit, covered with a fresh buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 20 min at 4 °C to allow DNA unwinding and the expression of alkali-labile-sites. Electrophoresis was performed for 20 min (25 V; 300 mA; 0.9 V/cm). Slides were then neutralized, washed in bi-distilled water and stained using a silver staining protocol (Nadin et al., 2001). After drying at room temperature overnight, gels were analyzed using an optical microscope. 100 cells were analyzed in microscopy and a damage class was determined to each cell. The damage classes were classified in: 0 = no tail (no damage); 1 = small tail smaller than the diameter of the head; 2 = tail length between one and two times the diameter of the head; 3 = long tail greater than twice the diameter of the head; 4 = long tail and more widespread than class 3. It was made a multiplication of each damage class by number of cells found in each damage class. The damage index (DI) was determined by the sum of these multiplications. The slides were analyzed under blind conditions at least by two different individuals.

2.4. Total homocysteine (tHcy) measurement

The tHcy levels in plasma were measured by liquid chromatography electrospray tandem mass spectrometry (LC–MS/MS), as described by Magera et al. (1999). This method is based on the analysis of 100 μL of plasma with 20 μL of homocysteine-d_8 (2 nmol) added as internal standard. After the step of reduction with 20 μL of 500 mM dithiothreitol followed by deproteinization, the analysis was performed in the multiple reaction monitoring mode in which tHcy and Hcy-d_4 were detected through the transition from the precursor to the product ion (m/z 136 to m/z 90 and m/z 140 to m/z 94, respectively). The retention time of tHcy and Hcy-d_4 was 1.5 min in a 2.5-minute analysis. The calibration was performed by a curve with 5 concentrations of Hcy. The results were expressed as μmol/L. In plasma, tHcy is the sum of free and protein-bound homocysteine, homocystine, and several other mixed disulfides.

2.5. Statistical analysis

Data were expressed as mean ± standard deviation. Comparisons between means were analyzed by unpaired Student's t-test or one-way ANOVA followed by Tukey test when the F value was significant, as appropriate. A p value lower than 0.05 was considered significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

3. Results

In this work we first evaluated the DNA damage in white blood cells from CBS-deficient patients under treatment and from healthy individuals (controls). We also investigated the in vitro effect of different concentrations of Hcy on DNA damage in white blood cells.

Tables 1 and 2 show the individual damage index (DI) values and the number of cells found in each damage class for the treated CBS-deficient patients and controls, respectively. We observed that CBS-deficient patients presented a higher number of cells with damage classes 2 and 3 than control group. Fig. 1 shows that DNA migration, and then, DNA damage, was significantly higher in the CBS-deficient patient group when compared to control group [$t(16) = 27.902$, $p < 0.001$]. The data reflect the increased number of cells with high damage class (classes 2 and 3) in leukocytes from CBS-deficient patients, as shown in Table 1.

Fig. 2 shows the in vitro effect of Hcy on DNA damage in white blood cells. We verified a concentration-dependent effect of Hcy on DNA damage [$F(4.10) = 906.436$, $p < 0.001$]. It can be seen in the figure that the lowest concentration of Hcy (10 μM) presented the lowest DI, whereas the highest concentration (300 μM) presented the highest DI. It is

Table 1
Age, individual DI values and number of cells found in each damage class in the homocystinuric patient group.

Patient	Age (years)	DI	Damage class				
			0	1	2	3	4
1	27	66	55	26	17	2	0
2	10	65	56	24	19	1	0
3	19	67	56	22	21	1	0
4	21	74	53	21	25	1	0
5	17	75	53	21	24	2	0
6	29	72	54	21	24	1	0
7	15	71	54	22	23	1	0
8	32	64	48	40	12	0	0
9	23	64	48	42	8	2	0
∑			477	239	173	11	0

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