

The effect of acute homocysteine administration on superoxide dismutase activity in young and aged rats

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Abstract

In mammals, dietary methionine is the only source of homocysteine (Hcy) whose metabolism depends on the availability of B12 and folic acid. Although Hcy is an essential metabolite, hyperhomocysteinemia is involved in aged-associated neurodegenerative disorders. The oxidative stress is the main ethiopathogenical mechanism of alterations induced by hyperhomocysteinemia. It is generally accepted that Hcy level increases, respectively the antioxidant protection decreases with aging.

We have studied the effect of Hcy acute administration on erythrocyte superoxide dismutase (SOD) activity. Wistar rats (20 young and 20 aged) have received a single intraperitoneal injection of saline (control) or Hcy (0.6 μmol/g body weight). Significantly lower levels of SOD activity (48153.23 U/gHb versus 87184.22 U/gHb; $p < 0.03$) were seen in young rats treated with Hcy versus control group. Lower levels, but not statistically significant were observed in aged rats.

In conclusion, hyperhomocysteinemia induces a significant decrease of SOD activity only in young rats.

Keywords: hyperhomocysteinemia, aging, SOD, oxidative stress

1. Introduction

Homocysteine (Hcy) is an endogenous sulfur-containing amino acid that is not available from the diet but is formed entirely from the metabolism of methionine [1] (Figure 1).

Methionine is the only essential, sulfur-containing amino acid found in the diet of mammals [1]. Methionine can also be generated by the methylation of homocysteine. Approximately 50% of homocysteine is re-methylated to methionine and 50% is transsulfurated to cysteine. Cysteine is a major source of glutathione in humans [2,3].

Methionine is supplied mainly by food proteins and is activated in the presence of adenosine triphosphate (ATP) and converted to S-adenosylmethionine (SAM).

SAM is a methyl donor in many trans-methylation reactions in humans. SAM is subsequently metabolized to S-adenosylhomocysteine (SAH) which is subsequently hydrolyzed to Hcy.

This transmethylation pathway is the only metabolic pathway that is known to produce Hcy. After formation, Hcy is either re-methylated into methionine or catabolised into cysteine (Cys) [3]. Methionine synthetase (MS) and its methyl-B₁₂ (i.e. methylcobalamin) catalyze the catabolism of Hcy via the re-methylation pathway. 5-Methyltetrahydrofolate (5-MTHF) donates a methyl group to MS which then transfers it to Hcy forming methionine.

Another route of Hcy re-methylation is mediated by betaine-homocysteine methyltransferase that utilizes betaine as a methyl donor. This minor pathway takes place mainly in the liver (Figure 1).

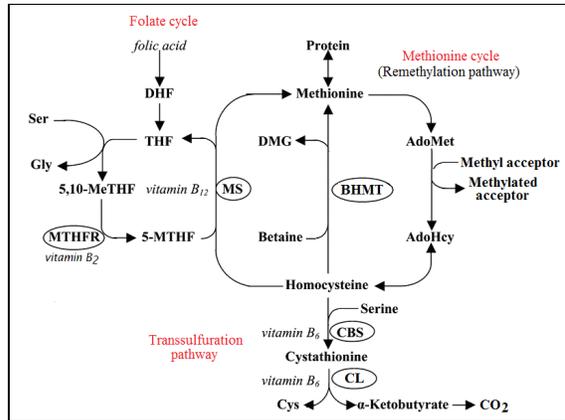


Figure 1. Homocysteine metabolism

MS = methionine synthase; **MTHFR** = methylene tetrahydrofolate reductase; **CBS** = cystathionine β -synthase; **CL** = cystathionine γ -lyase; **BHMT** = betaine homocysteine methyl transferase; **DMG** = dimethylglycine, **AdoMet** = adenosyl methionine; **AdoHcy** = adenosyl homocysteine; **DHF** = dihydrofolate; **THF** = tetrahydrofolate; **5-MTHF** = 5-methyl tetrahydrofolate

The transsulfuration pathway is in part mediated by cystathionine- β -synthetase (CBS). The active form of vitamin B₆, pyridoxal-5-phosphate, is the cofactor for CBS. Cystathionine is produced as a result of this process and it is in turn converted into cysteine and α -ketobutyrate, in the presence of cystathioninase, another B₆-dependant enzyme. Cysteine is further metabolized into glutathione which is very important for human antioxidant defense. When there is a methionine excess, e.g. after a meal (methionine loading), the transsulfuration pathway is favored. However, Hcy re-methylation to methionine is favored when there is a relative methionine deficiency in the cells, e.g. under fasting conditions. Hcy metabolism depends on the availability of B₆, B₁₂ and folic acid [4,5] as well as a number of enzymes [6].

Recent studies, both epidemiological and experimental, have shown that there are a number of factors that are considered to increase serum or plasma homocysteine levels in humans. Some are genetic and affect enzymes of homocysteine metabolism, in particular cystathionine beta-synthase (CBS) and methylen-tetrahydrofolate reductase (MTHFR).

There is some evidence that also links hyperhomocysteinemia with vegetarianism. Other lifestyle factors involved in hyperhomocysteinemia are smoking and coffee intake [7, 8].

Many publications have been reported that hyperhomocysteinemia is an independent risk factor for coronary heart disease [9], and the reducing of Hcy plasma level slows the progress of atherosclerosis [10]. In the last few years, evidence concerning homocysteine involvement in cardiovascular diseases and thrombosis has been accumulated [11-15]. Elevated Hcy levels can be found in neurodegenerative and neuroinflammatory diseases too [16].

It is generally accepted that Hcy level increases, respectively the antioxidant protection decreases with aging [17]. In order to verify whether high Hcy levels could alter oxidative stress markers, in the present study we evaluated the effect of acute Hcy administration on SOD activity in young and older rats.

2. Materials and Methods

Animals and reagents: In our experiment we used 40 female Wistar rats obtained from the Central Animal House of University of Medicine and Pharmacy, Cluj-Napoca, Romania.

Animals were maintained on a 12/12h light/dark cycle, in standard laboratory conditions. Twelve hours before the experiment a fasting period was kept with water ad libitum.

The injectable solution of Hcy (20.27 mg/mL) has been prepared freshly by solving D,L-Hcy (90%, TCI Europe nv Belgium) in sterile 0.9% NaCl solution.

Acute homocysteine administration: Four groups of rats, aged 1, respectively 12 months, were randomly formed, of 10 animals each (Table 1). They received a single intraperitoneal (i.p.) injection of saline (control) or saline Hcy. The injected amount was calculated such as to correspond to 0.6 μ mol/g body weight.

- I. Control group I: rats aged 1 month treated with 0.9 % NaCl solution
- II. Homocysteine group II: rats aged 1 month treated with Hcy
- III. Control group III: rats aged 12 months treated with 0.9 % NaCl solution
- IV. Homocysteine group IV: rats aged 12 months treated with Hcy

2h after injection, animals were sacrificed using an ethylic ether overdose and blood was drawn for further analysis.

SOD activity determination: Venous blood samples were collected in EDTA tubes. The whole blood (0.5 ml) was centrifuged for 10 minutes at 3,000 rpm and plasma was removed. The erythrocytes were washed three times with 0.9% NaCl solution, and then lysed by addition up to 2.0 ml cold redistilled water, followed by vigorous vortex-mixing and storage at +4°C for 15 minutes. The lysate was diluted with 0.01 mol/l phosphate buffer, pH 7.0, so that the % inhibition falls between 30% and 60%.

SOD activity was measured with RANSOD kit (RANDOX Labs., UK). This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2 - (4 - iodophenyl) - 3 - (4 - nitrophenol) - 5-phenyltetrazolium chloride (INT) to form a red formazan dye. The SOD activity is then measured by the degree of inhibition of this reaction. The activity was measured at 37°C on a Cobas Mira Plus (Roche), and absorbancy was monitored at 505 nm. The unit of activity is defined as the amount of enzyme that inhibits the rate of the formazan dye formation by 50%. The activity of SOD was expressed in U/L for absolute activity and U/gHb for specific activity. Hemoglobin concentration was determined by the Drabkin's method [18].

Statistical analysis: Data were analyzed by the Student's *t* test for unpaired groups. All analyses were performed using the SPSS software. Differences were considered statistically significant if $p < 0.05$.

Significantly lower level, given as arithmetic mean concentrations in U/gHb of SOD activity (48153.23 versus 87184.22; $p < 0.03$), were seen in Hcy treated rats versus control group aged 1 month and lower levels, but not statistically significant ($p < 0.3$), in rats aged 12 months. Older rats seem to be less affected than the younger rats. There is also no statistically significant ($p < 0.09$) difference of SOD activities determined in red blood cells between the control groups (Figure 2).

Table 1. The characteristics of the investigated groups

GROUPS		BODY WEIGHT MEAN (g) ± SE
I	Young Control	68.8 ± 8.87
II	Young treated with Hcy	60.6 ± 9.44
III	Aged Control	275.2 ± 10.71
IV	Aged treated with Hcy	285.5 ± 10.72
P	I vs. II	0.534975
	I vs. III	1.55E-11
	II vs. IV	5.74E-12
	III vs. IV	0.505392

SE- standard error

The mean value given for plasma Hcy in rats is normally $10.4 \pm 0.6 \mu\text{M}$ [19]. Hyperhomocysteinaemia generates a variable symptomatology including mental retardation, epilepsy, seizures and atherosclerosis, whose pathophysiology is poorly understood. Activated microglia by proinflammatory mediators may secrete a diverse range of neurotoxic factors such as NO and reactive oxygen species (ROS), which contribute to neuronal damage in neurodegenerative diseases [20, 21].

In this context, hyperhomocysteinaemia has been demonstrated to induce neuronal death, often associated with increased levels of ROS formation [22]. Indeed it has been previously demonstrated that chronic Hcy treatment induces oxidative stress in the cerebrum of rats, increasing lipid peroxidation and reducing enzymatic and non-enzymatic antioxidant defenses [23, 24].

The oxidative stress is the main ethiopathogenical mechanism involved in alterations induced by hyperhomocysteinemia. Hcy level increases, respectively the antioxidant protection decreases with aging [17].

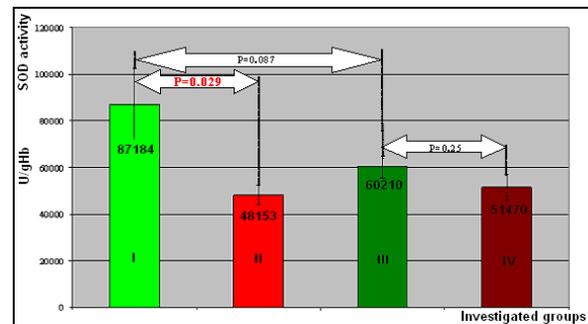


Figure 2. The effects of hyperhomocysteinemia on SOD activity

Hcy could undergo intracellular and extracellular autooxidation in the presence of molecular oxygen to generate ROS. The ROS, in turn, produce the actual damage by reacting with cellular constituents and small molecules such NO. Treatment of cultured endothelial cells with Hcy decreases bioavailability of NO and produces cytotoxicity mediated by hydrogen peroxide [25].

An alternative to the oxidative stress hypothesis is the molecular target hypothesis. Hcy itself can interact with specific molecular targets, either extracellular or intracellular [25]. The homocysteinilation reaction can lead to alteration of the biological function of multiple enzymes, receptors, growth factors and structural proteins [29]. The extent of formation of Hcy-protein derivatives is dependent on exposure time and Hcy concentration. It was reported that the longer the duration of exposure and the higher the concentration of Hcy, the greater the biochemical damage [30]. In this context, it was showed that SOD activity increased after treatment of rat aorta smooth muscle cells with D, L-Hcy (0-500 $\mu\text{mol/L}$) in a dose-dependent manner. In contrast, glutathione peroxidase activity decreased after Hcy administration, but there was no effect on catalase activity. It was observed that 5 mmol/L Hcy decreased steady-state mRNA for GPx by 90% [26].

In other paper it was reported that Hcy directly inhibits catalase breakdown of H_2O_2 by conversion of the enzyme into the inactive form [27]. Combined with the inhibition of GPx, Hcy is likely to be a major contributor to neurodegenerative pathology, which has been linked to oxidative stress in most cases [28].

In our experiment the short duration of exposure (2 hours) determined a decrease of SOD activity. In this context, previous studies indicate that Hcy administration can markedly decrease copper status in rats and, as a result, the level of erythrocyte SOD, a copper-dependent enzyme, is lowered in Hcy treated rats compared with control group [31].

In humans, plasma homocysteine increases linearly with age. This phenomenon is partly explained by the gradual decline in renal function. This increase is very gradual up to 60 years. From childhood to old age homocysteine levels approximately double [32]. After 60 years of age the increase is much faster, with a 1 $\mu\text{mol/l}$ increase for every 10 years of life. There is also a gender difference with men having approximately 2 $\mu\text{mol/L}$ higher levels than

women. However the gender difference becomes less visible with age, such that in elderly men and women the proportion of those with Hcy level higher than the upper reference range is similar [33].

4. Conclusion

In our hyperhomocysteinemia experimental model, we can see that the activity of red blood cell SOD was modified by Hcy, even after a short duration of exposure. As we expected, hyperhomocysteinemia disturbs the antioxidant balance by generating reactive oxygen species. The registered data show that in both groups (young and older rats) SOD activity decreases, the enzyme being a target for superoxide anion excessively produced.

Data revised and presented here allow us to argue that aging is a process directly related to systemic oxidative stress. Two elements of the oxidative stress have been recognized in human aging: a decrease in availability of nutritional molecular antioxidants and accumulation of products derived from the oxidation of biological structures. Moreover, supplementation with B_{12} and folic acid is necessary to provide healthy aging to the population, decreasing the occurrence of age-related diseases.

In summary, in the present study, we have observed that acute Hcy administration decreases the activity of SOD from red blood cells. However, many questions regarding the cellular mechanisms by which the acute administration of Hcy exerts this effect remain to be answered.

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