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Hyperhomocysteinemia's effect on antioxidant capacity in rats

Research Article

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Abstract: Hyperhomocysteinemia represents elevated homocysteine (Hcys) concentrations in blood above the normal range. In humans, the normal range of homocysteine is 5.0-15.9 mM/ml. High levels of homocysteine disturb the normal epithelial functions and correlate with cardiovascular diseases even at slightly increased concentrations. In homocysteine metabolism, vitamins play an important role. The mechanism through which homocysteine triggers these effects is not yet elucidated, but the involvement of reactive species may be the answer. It is not known whether the intra- or extracellular antioxidant system is more affected by elevated homocysteine levels. We studied the effects of hyperhomocysteinemia on the intra- and extracellular antioxidant defense systems in two different types of diet in rats. Type I was food with low folic acid and vitamin B12 content and type II was food with normal amounts of these two vitamins. Hyperhomocysteinemia was experimentally induced by oral administration of methionine 2 mg/kg body weight, single daily dose, for a 15-day period. Plasma concentrations of homocysteine were measured using an HPLC method. In the response of the intracellular antioxidant defense system against hyperhomocysteinemia, we determined the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in red blood cells, using RANDOX kits for manual use. For the extracellular response we determined the plasma total antioxidant status (TAS) also using a RANDOX kit for manual use. Our data show that methionine load induces hyperhomocysteinemia despite normal vitamin supply in rats. SOD activity rose with simultaneous decrease in GPx activity independently of diet; this might suggest that the intracellular defense system was disturbed by the rise in homocysteine level. TAS decrease suggests that the extracellular antioxidant defense was also affected. We assume that hyperhomocysteinemia is directly linked to reactive species generation and the intracellular space seems to be more affected than the extracellular one.

Keywords: *Methionine • Hyperhomocysteine • TAS • SOD • GPx*

1. Introduction

In the last period, evidence concerning homocysteine involvement in thrombosis and cardiovascular diseases has been accumulated [1-9].

Homocysteine occurs as a result of methionine metabolism, which compulsorily requires the presence of vitamins such as B_6, B_{12} and folic acid. Some genetic polymorphisms of methyltetrahydrofolate reductase and cystathionine synthetase, as well as inappropriate supplies of folic acid (B_9), pyridoxine (B_6), or cyanocobalamine (B_{12}), cause a decrease in methionine metabolism and, as a consequence, an increase in homocysteine blood concentration [10-15].

Even though the mechanisms through which high levels of homocysteine generate thrombosis and cause cardiovascular events are not clear, the possible involvement of reactive species should be considered [16-18].

At the cellular level oxidative stress represents an important decrease in the reducing capacity of oxidation-reduction couples [19]. The effects of oxidative stress

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depend on the intensity and size of the event. In mild stress cells can overcome little disorders and return to the normal state, whereas severe stress can trigger cellular death [20].

Cellular defense against reactive species is conducted through intracellular systems such as superoxide dismutase, catalase, glutathione peroxidase, peroxiredoxine, or small molecules that act as reducing agents such as ascorbic acid (vitamin C), tocopherol (vitamin E), vitamin A and glutathione.

In the extracellular space the same antioxidant molecules and a few others – such as uric acid, bilirubin, albumin and metal-binding proteins (for example, ferritin or ceruloplasmin) – operate to preserve the oxidative balance. All these circulating species acting as reducing agents are together known as total antioxidant status [21].

It is not known whether the intra- or extracellular antioxidant system is more affected by elevated homocysteine levels. Homocysteine is metabolized at a high rate in red blood cells, so determining the activity of the intracellular antioxidant defense system within erythrocytes can provide a way to appreciate the responsiveness of the intracellular medium at an elevated homocysteine level.

We studied the effects of hyperhomocysteinemia on the intra- and extracellular antioxidant defense systems in rats using two different types of diets with regard to vitamin content.

Hyperhomocysteinemia was experimentally induced by oral administration of methionine 2 mg/kg body weight, single dose daily, for a 15-day period. Homocysteine concentration was determined by an HPLC method [22].

In order to investigate the response of the antioxidant systems to high levels of homocysteine, the activities of intracellular superoxide dismutase (SOD), glutathione peroxidase (GPx) and plasma total antioxidant status (TAS) were determined using RAnDOx kits for manual use.

Our data show that administered doses of methionine induced hyperhomocysteinemia in rats despite the presence of vitamins. SOD activity rose with simultaneous decrease in GPx activity, regardless of the animal's diet. This might suggest that the intracellular defense system was affected by the rise in homocysteine level. Plasma TAS concentration decreased in both groups as a result of high plasma homocysteine levels.

In conclusion, our data show that high levels of homocysteine are correlated with decreases in the extra- and intracellular antioxidant defense capacity, and the intracellular space seems to be more affected than the extracellular one.

2. Material and Methods

2.1. Materials

All reagents for the determination of plasma homocysteine concentration were for HPLC use, acquired from Fluka. The antioxidant activity of intra- and extracellular systems was measured using RAnDOx kits for manual use, acquired from RAnDOx.

2.2. HPLC

Homocysteine plasma concentration was determined using the HPLC method for human plasma [22]. We used tri-butylphosphine (TBP) instead of tri-n-butylphosphine (TnBP) as the reducing agent for oxidized and protein-bound homocysteine. The derivatisation agent was 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT) [23].

The chromatographic system consisted of Agilent 1200 HPLC 6520, Binary Pump, Zorbax SB-C 18 (4,6mm x 250mm) (5μm) and Detector UV-VIS (DAD). Elution was realized in gradient mode [22]. Sample volume was 20 μl, flow rate 1,2 ml/min, wavelength was set at 355 nm and column temperature was maintained at 25^{0}C.

2.3. Sample preparation

The following amounts were added: 0.3 ml of rat plasma, 0.3 ml of 0.2M phosphate buffer pH 7.6, and 30 μl of 10% TBP in methanol. The mixture was heated for 30 minutes at 60^{o}C. After cooling at room temperature, 30 μl of 0.1M CMQT and 0.3 ml of 3M perchloric acid (PCA) were added. After centrifugation at 12000 rpm for 10 minutes, an aliquot of 20 μl of supernatant was injected into the column.

2.4. Calibration curve

A calibration curve was realized in order to determine homocysteine levels in rat plasma. The homocysteine stock solution was prepared at a concentration of 10 mM; working solutions were prepared by appropriate dilution of the stock one. Working solutions in the range of 1-32 μmol/ml were added to 0.3 ml of rat plasma and processed as described above.

2.5. Experimental procedures

The experiment was performed on two groups of 10 adult Wistar male rats, each weighing 150-200 g. Hyperhomocysteinemia was experimentally induced by oral administration of a single daily dose of methionine, 2 mg/kg body weight. All experiments were performed according to the standards of the European legislation concerning the care and use of experimental animals.

Figure 1. Chromatograms of plasma samples obtained from the same rat, initially (a) and after methionine administration (b).

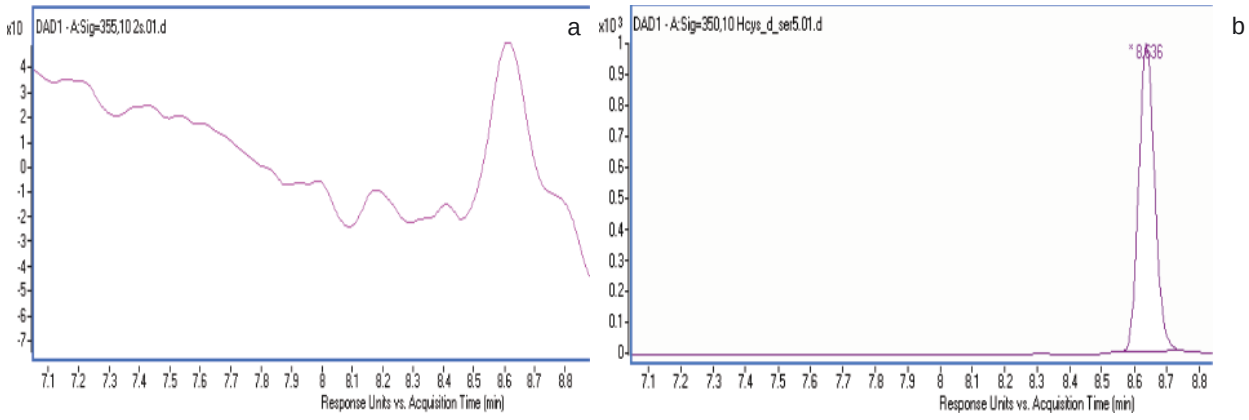

Table 1. Concentration of homocysteine in rat plasma determined initially and after methionine administration for a 15-day period.

	Homocysteine concentration (μM) Mean ±SD	
N = 10 animals/series	Initial	Final
Group I (animals receiving vitamin-deprived food exposed to methionine loading)	12,96±1.03	>32
Group II (animals receiving standard food and exposed to methionine loading)	11,33±0.97	>32

The animals from the two groups were fed differently, as follows:

- Group **I** received food deprived of folic acid and vitamin B_{12}, but containing apples 5g/100g animal, carrots 5g/100g animal, black bread 10g/100g animal and barley 10g/100g animal, for 15 days. Meanwhile, animals were administered methionine 2 g/kg body weight as a single daily dose by oral route.

- Group **II** received standard food containing 0.5 mg/kg body weight folic acid and 10 mg/kg body weight vitamin B_{12} for 15 days. Meanwhile, these animals were also administered methionine 2 g/kg body weight as a single daily dose by oral route.

Samples of blood were taken in order to determine homocysteine plasma concentration and oxidative stress parameters, initially and after 15 days of methionine exposure.

Total homocysteine was determined in the rat plasma using an HPLC method.

In order to investigate the response of the antioxidant systems against high levels of homocysteine, the activities of intracellular superoxide dismutase (SOD), glutathione peroxidase (GPx) and plasma total antioxidant status (TAS) were measured using the Randox kit for manual use.

2.6. Statistical analysis

The statistical analysis for all determined parameters was realized by comparing the two groups using the analysis of variance (AnOVA-one-way); a coefficient of $p<0.05$ was considered to indicate a statistically significant difference between groups.

3. Results

The calibration curve for homocysteine was linear in the range of 1-32 μM. The linear regression equation was Hcys μM = 30.97 X – 8.9676 with a correlation coefficient r^2=0.9975. The coefficient of variation in Hcys determination for 5 replicates was within 5%. Retention time for Hcys was 8,626 ±0,008 minutes. Chromatograms obtained from rat plasma elution initially and after methionine loading are presented in Figure 1.

Homocysteine was efficiently separated from other substances with similar structure. Starting from minute 7.1 to minute 8.8 (the interval during which homocysteine elutes) no interfering compounds were observed.

Homocysteine levels determined in rat plasma initially and after methionine administration are presented in Table 1.

Our data show that in both groups homocysteine concentrations are significantly increased after 15 days

Table 2. Total antioxidant status determined initially and after methionine administration for a 15-day period.

	TAS (mmol/l plasma) Mean ±SD	
N = 10 animals/series	Initial	Final
Group I (animals receiving vitamin-deprived food and exposed to methionine loading)	0.908±0.022	0.760±0.024
Group II (animals receiving standard food and exposed to methionine loading)	0.897±0.036	0.756±0.041

Table 3. Superoxide dismutase activity, in rat red blood cells, determined initially and after methionine administration for a 15-day period.

	SOD (U/ml whole blood) Mean ±SD	
N = 10 animals/series	Initial	Final
Group I (animals receiving vitamin-deprived food and exposed to methionine loading)	202.88±16.7	944.7±40.1
Group I (animals receiving standard food and exposed to methionine loading)	195.30±17.2	945.7±42.1

of daily methionine administration when compared with the initial measurement; in fact homocysteine concentration exceeds the upper concentration of the linearity domain.

Homocysteine concentration presents at similar levels for both groups, despite the fact that group II received standard food that contained the appropriate amounts of folic acid and vitamin B_{12}. It is known that an appropriate vitamin supply is a way of decreasing homocysteine levels in humans.

For group II, we expected homocysteine levels to remain at least at the same level as in the beginning of the experiment.

We assume that repeated methionine administration exceeds by far its metabolisation despite normal vitamin supply, and that might explain the hyperhomocysteinemia that also manifested in group II. The vitamin amount in standard food fails to lower homocysteine levels. Recent literature information is consistent with our data concerning the lack of benefit from folate supplementation [24]

Statistical analysis (AnOVA-one-way) indicated significant differences within groups (*$p < 0.0001$, when compared to the initial measurement), but no significant differences between groups.

The total antioxidant status determined in rat plasma is presented in Table 2.

TAS levels were significantly decreased in both groups. As expected, severe hyperhomocysteinemia disturbs the antioxidant balance by generating reactive species and decreasing the total antioxidant capacity in the extracellular space.

Statistical analysis (AnOVA-one-way) indicated significant differences within groups (*$p < 0.0001$ when compared to initial measurement) and no significant differences between groups ($p= 0.9262$ for group I versus group II in the initial measurement, and $p= 0.9964$ for group I versus group II in the final one).

The superoxide dismutase and glutathione peroxidase activities, determined in rat red blood cells, are presented in Tables 3 and 4 respectively.

SOD activity increased in both groups. SOD is an enzyme involved in reactive species detoxification and the increased activity might indicate a rise in the concentration of these species, which in our case seems to have been generated by a prolonged exposure to hyperhomocysteinemia.

Statistical analysis (AnOVA-one-way) indicated significant differences within groups (*$p < 0.0001$ when compared to initial measurement), and no significant differences between groups ($p= 0.975$ for group I versus group II in the initial measurement, and $p= 0.9998$ for group I versus group II in the final one).

GPx activity decreased significantly in both groups. The decrease in GPx activity can be explained by a supposed decrease in glutathione concentration, the main cofactor of this enzyme. The increased amount of reactive species consumed more glutathione, and the diminished glutathione concentration led to decreased GPx activity. We assume that prolonged exposure to

Table 4. Glutathione peroxidase activity, in rat red blood cells, determined initially and after methionine administration for a 15-day period.

	GPx (U/L haemolysate) Mean ±SD	
N = 10 animals/series	Initial	Final
Group I (animals receiving vitamin-deprived food and exposed to methionine loading)	60211±4926	34259±3897
Group II (animals receiving standard food and exposed to methionine loading)	64254±5067	39835±3524

hyperhomocysteinemia generated reactive species that disturbed the antioxidant balance.

Statistical analysis (AnOVA-one-way) indicated significant differences within groups (*$p < 0.0001$ when compared to initial measurement), and no significant differences between groups ($p= 0.4029$ for group I versus group II in the initial measurement, and $p= 0.1568$ for group I versus group II in the final one).

4. Discussions

Our data show that hyperhomocysteinemia was installed extremely rapidly, after only 15 days of methionine administration. Despite choosing an adequate methionine diet of 2.22 g/kg [25], high concentrations of homocysteine were found after a short period of time.

It was expected that standard food, containing the appropriate vitamin amount, would lower or at most moderately increase homocysteine levels. Instead, the increase in homocysteine levels was as high as in the cases with vitamin-deprived food.

Homocysteine is metabolized within cells by two processes: remethylation and trans-sulfuration [26,27]. The fact that homocysteine levels remained high despite a normal vitamin supply suggested that there was another process that was at least as responsible as vitamin deficiency for increasing homocysteine levels. We assume that the high homocysteine level found in both groups might have been generated as a result of disturbances in the trans-sulfuration pathway.

TAS levels were significantly decreased no matter what the animal diet was. It is known that auto-oxidation of excess homocysteine produces free toxic radicals [4,16,19] and as a consequence, decreased TAS levels are to be expected. The decay in TAS, as significant as it was, was not as severe as the changes observed in intracellular enzyme activity.

The activity of SOD, an enzyme activated by the presence of superoxide radicals, suffered a huge increase of about four times the initial values; this situation could be explained by a significant rise in the amount of reactive species. On the other hand, the increase in reactive species concentration usually activates GPx and as a consequence we expected an increase in GPx activity. In fact, GPx activity decreased to almost half of its initial activity. This result can be explained by a severe decrease in intracellular glutathione, the main cofactor of this enzyme, leading to the observed decay in GPx activity. In addition, the decrease in GPx activity triggered reactive species accumulation, which determined a supplementary increase in SOD activity.

Hyperhomocysteinemia, once installed, led to a severe imbalance in the intracellular antioxidant defense systems.

Our findings that hyperhomocysteinemia deeply affects the intracellular antioxidant systems are supported by the fact that homocysteine is mainly metabolized within the cell [28,29].

In conclusion, hyperhomocysteinemia is directly linked to reactive species generation and the intracellular space seems to be more affected than the extracellular one.

5. Conclusions

The obtained data show that methionine administration in doses of 2 mg/kg b.w. induced hyperhomocysteinemia, even with a normal vitamin supply diet in rats. This suggests the involvement of different mechanisms apart from vitamin deficiency, and trans-sulfuration might be one of them.

Hyperhomocysteinemia is directly linked to reactive species generation and the intracellular space seems to be more affected than the extracellular one.

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