

## Decreased Activity of Thiol Peroxidase (Glutathione Peroxidase 3) in Blood Plasma as an Indicator of Aging

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### Abstract

Thiol peroxidases is a superfamily of antioxidant enzymes catalyzing the thiol-dependent reduction of hydroperoxides. Thiol peroxidases of glutathione peroxidase (Gpx) family utilize reduced glutathione (GSH) as a reductant. Early studies recognized that plasma selenium-dependent Gpx (Gpx3) possesses a wide thiol specificity. Our previous study statistically confirmed the ability of rat Gpx3 to utilize reduced homocysteine (Hcy-SH) instead of GSH. Thus, GSH can be completely replaced by Hcy-SH in a reaction mixture, that allows to determine Gpx3 activity with higher selectivity. In present study, the hypothesis of the age-associated decline of thiol peroxidase activity in rat plasma was tested using Hcy-SH as a thiol substrate. The enzymatic activity in 23-24-month-old rats was significantly lower than in 6-8-month-old rats ( $p=0.0012$ , Wilcoxon-Mann-Whitney test). These data are in agreement with the results obtained by other authors revealed the diminished total Gpx activity in serum of 24-month-old rats. The progressive aging-associated decline of Gpx activity in human plasma is also known. Gpx3 activity in plasma may be an appropriate indicator of aging process due to its relatively high value in adult, not aged animals and humans, and its subsequent prominent aging-specific decline.

**Keywords:** Aging; Glutathione peroxidase; Homocysteine; Selenium

### Introduction

A feature of aerobic metabolism is the production of reactive oxygen species, in particular, superoxide anion-radical, the dismutation of which leads to hydrogen peroxide formation. Hydrogen peroxide may react by free radical mechanisms with peroxidation of biomolecules; the intensification of this process takes place in development of various diseases and aging [1].

Thiol peroxidases are antioxidant enzymes which include glutathione peroxidases (Gpx) and peroxiredoxins. Enzymes of these two families catalyze thiol-dependent reduction of inorganic ( $H_2O_2$ ) and organic hydroperoxides [2]. Glutathione (GSH) is a source of reducing power for Gpx [3]. Among human Gpx, eight forms are known and five of which (Gpx1,2,3,4, and 6) are selenium-dependent [4].

In 1998, a detailed study was published [5], in which the authors determined the levels of oxidative protein damage and lipid peroxidation in rat tissues during aging, and the activities of antioxidant enzymes, particularly superoxide dismutase (SOD) and Gpx, were also determined. An interesting finding was the gradual decrease of SOD activity in serum from 1-month-old to 24-months-old rats, while Gpx activity in serum remained relatively high after age of 6 months and was significantly decreased only in serum from 24-month-old rats [5]. In general, it can lead to suggestion that the activities of SOD and Gpx in serum or plasma (and also the ratio between them) may be predictors of the aging process.

In paper [5], the authors found the statistically significant difference in serum Gpx activity when 24-month-old rats were compared with 6-

month-old rats, with p-value less than 0.01. This is a high significance level, but, nevertheless, it is not the fact, that the I type error probability is very small, because the multiple comparisons were evidently made. Moreover, the Student's test was applied while the data evidently contain the samples with different variances [5]. In such cases, the repeated studies are helpful to resolve doubts. Thus, I decided to check the hypothesis, that plasma Gpx activity in aged rats is diminished in comparison with that in middle-aged rats.

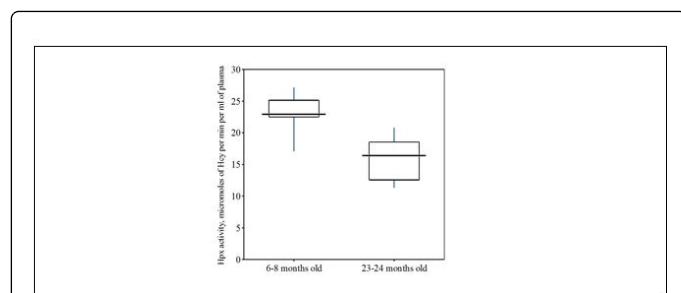
The Gpx activity in plasma or serum can easily be interpreted as the activity of Gpx3, the extracellular Gpx, also called plasma or serum Gpx [6]. However, in plasma of rodents (mice), about 9% of the total Gpx activity belongs to Gpx1, not to Gpx3 [7]. While Gpx1 is highly specific to GSH, the Gpx3, in contrast, has a wide thiol specificity and able to use cysteine, thioredoxin and glutaredoxin as sources of reducing power [4,8]. Recently, we confirmed (with high probability) that Gpx3 is able to use homocysteine (Hcy-SH) as a reductant instead of GSH [9], and, thus, GSH can be completely replaced by Hcy-SH in a reaction medium, that allows to avoid the participation of Gpx1 in reaction process. The proportions between Gpx3 and Gpx1 activities in plasma of rats of various age are unknown and may be different, therefore, I used Hcy-SH as a reductant in the present study and, thus, determined homocysteine peroxidase (Hpx) activity to make sure, with high probability, that the activity I determined belongs to Gpx3, not to the mixture of two different Gpx forms.

Wistar rats were donated by Rappolovo breeding farm and kept in the vivarium of DO OTT Institute of Obstetrics, Gynecology and Reproductology, St Petersburg, under standard laboratory conditions (12:12 hr light-darkness regimen, rat pellets and water ad libitum). The female offsprings of donated rats, which were born and reared in the vivarium to age of 6-8 and 23-24 months, were used in the study. In the 2nd half of dayphase rats were narcotized with chloroform and

decapitated; blood was collected with Vacuette K3E tubes (GBO, Austria), plasma was separated by centrifugation at 1600×g during 15 min at room temperature and then stored at -85°C for a few weeks.

Hpx activity was determined using the Ellman's-reagent-based method adopted for rat plasma and described in paper [9]. Chemicals used are listed in papers [9,10]. Reagent A: 0.532 mM thiol (DL-Hcy-SH) in a mixture prepared by mixing of sodium azide aqueous solution (26 mg/mL) with 0.1 M tris-HCl buffer (containing 0.34 mM EDTA, pH 8.5) in a volume ratio of 1:17. Reagent B: 2 mM aqueous solution of H<sub>2</sub>O<sub>2</sub>. Using reagents, A and B, the procedures described below result in 0.460 mM thiol and 0.077 mM H<sub>2</sub>O<sub>2</sub> in a mixture for enzymatic reaction. Biological material was the rat plasma diluted 20-fold by physiological saline (0.9% NaCl). Reagent A (360 µL) was preincubated during several min at 37°C, then 40 µL of biological material (or its diluent-for estimation of non-enzymatic reaction) was added. Simultaneously with the biological material (or with its diluent) and without preliminary mixing, 16 µL of reagent B was added. After 40 s, 80 µL of 30% trichloroacetic acid (w/v) was added to terminate the reaction. A few minutes later after the trichloroacetic acid was added to mixture, tubes with mixture were centrifuged at 1000×g for 10 min. The residual thiol concentration was determined in supernatant by the Ellman's reagent [9,11], the absorbance was read at 412 nm using DU-65 spectrophotometer (Beckman Coulter, USA). Thiol concentration was determined using the 2-nitro-5-thiobenzoate extinction coefficient of 14,150 M<sup>-1</sup> × cm<sup>-1</sup> (taking into account the dilutions and the 1:1 stoichiometry between thiol and 2-nitro-5-thiobenzoate [12]).

Enzymatic activity was counted by subtracting the rate of non-enzymatic reaction from the total reaction rate and was expressed as micromoles of Hcy-SH consumed per min per mL of plasma. To describe the samples, the median values, quartiles and the lowest and highest values were used (plotted in Figure 1). The nonparametric Wilcoxon-Mann-Whitney test was used for statistical comparison of the two samples. The exact probability of type I error was calculated with R (version 2.13.1) [13].



**Figure 1:** Hpx activity in blood plasma of rats aged 6-8 and 23-24 months. N= 9 and 7, respectively. The initial concentrations of Hcy-SH and H<sub>2</sub>O<sub>2</sub> in reaction mixture were 0.46 and 0.077 mM, respectively; pH 8.5; temperature was 37°C. The line crossing the box represents the median, the lower and the upper boundaries of the box are the first and third quartiles, and the «whiskers» are the lowest and highest values. Differences between the two groups are significant (p=0.0012, Wilcoxon-Mann-Whitney test).

The results (Figure 1) demonstrate an age-associated decrease of Hpx activity in blood plasma of rats and, thus, confirm the data obtained earlier for total Gpx activity in rat serum [5]. The hypothesis of the diminished activity of Gpx3 in plasma of aged rats is also

confirmed in the present study using Hcy-SH (instead of GSH) as a thiol substrate in thiol peroxidase reaction. Median values were of 22.97 and 16.45 for 6-8-month-old and 23-24-months-old rats, respectively. Hpx activity is decreased 1.4-fold when medians are compared, that is similar to results in paper [5] (the detailed plot analysis of in paper [5], in which the plotted mean values of serum Gpx activity are presented, reveals 1.46-fold decrease of activity from 6-month-old rats to 24-months-old rats).

Blood sampling is a simple procedure which can be performed without any serious damage to donor. In view of this, the testing blood components for indicators of aging process (if they are really informative) is much more preferable than biochemical testing the other internal tissues or organs.

Comparing the age-associated dynamics of Gpx activity with that of SOD activity in serum (plasma) [5], it can be proposed that serum (plasma) Gpx activity is better indicator of senescence than SOD activity. SOD activity decreases gradually, and the decrease from age of 1 to 6 months seems to be most prominent, while 6-month-old rats are certainly not old rats. Gpx activity increases from age of 1 to 6 months, remains relatively stable from age of 6 to 18 months, and then declined in 24-months-old rats [5]. According to our developmental observations, the serum (plasma) Gpx (Hpx) activity increased to the adult levels already in 1.5-2-month-old rats and then remains relatively high for a few months (Razygraev and Taborskaya, data in processing). Therefore, in case of certainly adult, healthy, and not aged animal, we expect to observe a high level of Gpx (Hpx) activity in plasma or serum.

In humans, the similar age-associated dynamics of plasma (serum) Gpx activity was found earlier: comparing mean values, Gpx activity decreased less prominently (1.26-fold) from age of 15-30 to 46-65 years [14] and more prominently (1.8-fold) from age of 50-60 to more than 80 years [15]. It is appropriate to mention, that selenium is essential for Gpx3 biosynthesis [16]. Thus, in a simple word, if we reveal prominently diminished serum (plasma) Gpx (Hpx) activity in adults, we can suggest the physiological senescence, age-associated diseases, accompanied by decrease of Gpx activity, and/or insufficient selenium consumption. Discussed facts, taken together, are in concordance with the data showing the positive correlation of selenium distribution with longevity [17]. Probably, the mechanisms supporting Gpx3 biosynthesis undergo involution in aging individuals earlier if the selenium consumption is regularly insufficient.

Gpx3 was classified as one of the selenoproteins whose activities and concentrations are most sensitive to selenium deficiency [18]. To use Gpx3 activity in plasma as an indicator of aging, it can be proposed to exclude the short-term insufficient selenium consumption as a cause of diminished Gpx3 activity. An evaluation of Gpx3 activity in relation to the background selenium status seems to be preferable. Obesity may be associated with Gpx3 decrease in plasma [16], therefore this condition may limit the use of plasma Gpx3 activity as an indicator of senescence. In cases of already developed some oncological diseases, accompanied by down-regulated Gpx3 (e.g. prostate cancer [16]), the use of Gpx3 activity in plasma as an indicator of aging may be redundant, because manifestations of these diseases are often aging-associated by themselves [19].

In humans, approximately 40 µg of selenium per day achieved maximal Gpx activity in plasma [20]. To maintain the Gpx3, the sufficient amount of the products, in which the selenium is commonly abundant and highly bioavailable (primarily wheat and meats, also

fish, garlic, onions, broccoli, legumes, nuts, and several other products-reviewed in [20] and [21]), can be recommended to include in the diet. At the absence of selenium supplementations in these products, the geographical origin of them may be taken into account (some regions are selenium-deficient [17,21]). Direct consumption of nutritional supplements or 'nutraceuticals' based on selenium may also be effective. An example of the supplement is the selenium-enriched yeast. Data on toxicity of selenium from selenized yeast are scarce [21] that makes this supplement preferable. Certain preparations with antidiabetic effects (thiazolidinediones) are known to increase biosynthesis of Gpx3 [22] and may be tested for their ability to reverse the aging-associated decline of plasma Gpx3 activity. All these ways to increase the Gpx3 activity, when considered for aging human, are mostly theoretical and need further testing in context of studying Gpx3 during aging.

The focus on Hpx activity of Gpx3 is of particular interest in context of discussion of aging process. The older age is associated with elevated Hcy concentration in blood [23]. Total Hcy represents various forms of this amino acid [24], and it is not excluded that these forms are different in their toxic effects. Hpx reaction includes conversion of Hcy-SH to Hcy disulfide (Hcy-SS-Hcy). If these two forms of Hcy are not equally toxic, Gpx3 may be a regulator of toxicity of age-associated hyperhomocysteinemia [9,10]. In view of this, Gpx3 may become a target for nutritional and/or pharmacological treatment.

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