Arch. Biol. Sci., Belgrade, 62 (3), 589-594 , 2010 DOI:10.2298/ABS1003589M

GLUTATHIONE STATUS IN THE BLOOD OF RATS AFTER RETICULOCYTOSIS INDUCED BY PHENYLHYDRAZINE AND BLEEDING

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Abstract - In this experiment, we compared the *in vivo* effects of phenylhydrazine (PHZ) and bleeding treatment on the redox status and glutathione antioxidative mechanism parameters in the plasma and red blood cells (RBC) of rats. Results showed a lower level of reactive oxygen species (ROS), a higher level of lipid peroxidation and the effective antioxidative role of the glutathione system in the blood of bleeding rats. PHZ-treatment induced higher concentrations of ROS and an accumulation of oxidized glutathione in the plasma, while the glutathione system showed a satisfactory antioxidative capacity in the RBC of rats. When comparing the two anemic groups, the PHZ-treated rats showed marked oxidative stress in the plasma.

Keywords: Bleeding, blood, glutathione, phenylhydrazine, rats.

UDC 616.155:59.084:547.556.8

INTRODUCTION

Anemia is usually induced by bleeding or PHZ treatment under experimental conditions (Rapoport, 1986). Successive bleeding of animals or humans causes the stimulation of erythropoiesis and as a consequence there is an increase in the number of reticulocytes in peripheral blood. PHZ-induced anemia is the result of rapid hemolysis due to damage and the complex interactions between PHZ and red blood cell (RBC), proteins and lipids (Stern, 1989; Fukushima and Kon, 1990).

Oxygen is a primary oxidant in metabolic reactions which are required in order to obtain energy by oxidizing various organic molecules. Oxidative stress is a consequence of these reactions and can be defined as an impaired balance between the development of reactive oxygen species (ROS) on the one hand and antioxidant defense mechanisms on the other hand (Halliwell and Gutteridge, 1999). The redox state of a biological system is kept within a narrow range under normal conditions. Cells and tissues have the mechanisms to restore the redox state after temporary exposure to high concentrations of ROS.

The most important mechanisms of redox homeostasis is based on the ROS-associated induction of a redox-sensitive signal cascade that leads to a higher expression of antioxidative enzymes or the elevated intensity of the cysteine transport system, which maintains the high level of glutathione in cells. Since mitochondria are a major site of free radical generation, they are highly enriched with antioxidants including glutathione (GSH) and enzymes, such as Mn-containing superoxide dismutase (Mn SOD) and glutathione peroxidase (GSH-Px), which are presented on both sides of their membranes in order to minimize oxidative stress in the organelle (Halliwell and Gutteridge, 1999).

The induction of oxidative stress and damage has been observed following exposure to various xenobiotics, one of them being PHZ.

In order to induce high reticulocytosis, since reticulocytes are a valuable source of information for many of the metabolic pathways and especially the oxidative stress response system, we experimentally induced anemia by successive daily bleeding and by PHZ-treatment, both of which are the most common procedures. The goal of this study was to compare the redox state, oxidative damage and glutathione antioxidative mechanisms in the plasma and RBC in bleeding- and PHZ-induced anemia in rats.

MATERIALS AND METHODS

Chemicals

PHZ, chemicals for solutions and enzymes were obtained from Sigma (St.Louis, MO, USA) and Merck (Darmstadt, Germany).

Animals, RBC collection and incubation

In this study, the RBC of rats (*Wistar* albino, male, 250-350 g body mass) were used. The animals were kept at 21 ± 1 ^{0}C and exposed to a 12 h light – 12 h dark cycle. All rats were housed in individual cages and given a standard diet and water *ad libitum*. Control groups were obtained from untreated (I) and 0.9% NaCl-treated (0.5 ml 0.9% NaCl for three days) rats (II). Reticulocytosis was induced by PHZ treatment (35 mg/kg body weight for 3 days) (III) and by daily bleeding of the rats (1.5-2 ml of blood from the tail vein) for 8 days (IV). After 7 (I, II and III) and 9 days (IV) the rats were anaesthetized by ether and blood was taken by exsanguination. Blood was collected in tubes containing heparin.

For redox status determination the collected blood was centrifuged for 10 min at 5000 rpm, the plasma was separated, while the RBC were washed three times with NaCl 0.9%. Washed-out erythrocytes were lysed with dH_2O (1:3, v/v) at 0^{o}C for 30 min. All samples were extracted from plasma and lysate. After extraction the samples were stored at -80^{o}C until analysis (in less than 1 month).

Evaluation of ROS concentrations

The concentrations of ROS were determined after extraction using the following protocol: ½ vol 3 M perchloacetic acid and 2 vol of 20 mM EDTA were added to 1 vol of plasma. After extraction on ice (15 min) and centrifugation for 4 min/15 000 rpm the extracts were neutralized using 2 M K_2CO_3.

The spectrophotometric determination of the superoxide anion ($O_2^{\cdot-}$) was based on the reduction of nitroblue tetrazolium (NBT) in the presence of $O_2^{\cdot-}$ (Auclair and Voisin, 1985). The determination of the hydrogen peroxide (H_2O_2) concentration was based on the oxidation of Phenol Red (PR) in the presence of Horse Radish Peroxidase (HRPO) as a catalyst (Pick and Keisari, 1980).

Evaluation of lipid peroxide level

The level of lipid peroxidation products was determined on the basis of the reaction of lipid peroxidation products (malondialdehydes) using the Ohkawa method with thiobarbituric acid (thiobarbituric acid reactive substances - TBARS) (Ohkawa et al., 1979).

Evaluation of glutathione level

The level of reduced glutathione (GSH) was determined on the basis of GSH oxidation with 5.5-dithio-bis-6.2-nitrobenzoic acid using the Beutler method (1975a) and the concentration was expressed as nmol/ml plasma (RBC). Concentrations of oxidized glutathione (GSSG) were determined enzymatically by glutathione reductase using the Beutler method (1975b) after inhibition of GSH oxidation by N-ethylmaleimide. The level of GSSG was expressed as nmol/ml plasma (RBC).

Evaluation of AOS enzymes activity

The antioxidative enzymes activities were determined in lysate.

Glutathione peroxidase (GSH-Px) activity was assayed following the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) with t-

butyl-hydroperoxide as a substrate (Maral et al., 1977). The activity of glutathione reductase (GR) was determined using the method of Glatzle et al. (1974). The method is based on the capacity of GR to catalyze the reduction of GSSG to GSH using NADPH as a substrate. Glutathione-S-transferase (GST) activity for 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate was measured according to the method of Habig et al. (1974). The activities of GSH-Px, GR and GST were expressed in U/ml RBC.

Statistical analysis

All values are expressed as mean ± SEM. Statistical evaluation was calculated by one way ANOVA. For all comparisons, $p < 0.05$ was considered as significant.

RESULTS

The effects of PHZ and successive bleeding treatment on the redox status were evaluated in the plasma and RBC of Wistar albino rats. The obtained results showed that all parameters followed for the placebo control (II) were not different compared to the control (I).

The ROS levels in the plasma of the groups of animals investigated are shown in Fig. 1. The concentration of $O_2^{\cdot -}$ was significantly higher in the plasma of the PHZ-treated rats, compared to the control (I) and bleeding groups (IV) (Fig.1). H_2O_2 concentration was significantly higher in the plasma of the PHZ-treated rats (III), compared to the control (I) (Fig. 1).

The TBARS level, an indicator of oxidative damage processes, was significantly higher in the plasma of the bled rats (IV), compared to the control (I) (Tab. 1). In the plasma of the PHZ-treated rats, the GSH level was significantly lower, which was followed with a high GSSG and GSSG/2 GSH ratio, compared to the control group (I) (Tab. 1). The results for the bled rats (IV) were reciprocally different – the GSH level was significantly higher, which followed with lower concentration of the GSSG and GSSG/2 GSH ratio, compared to the PHZ treated group (I) (Tab. 1).

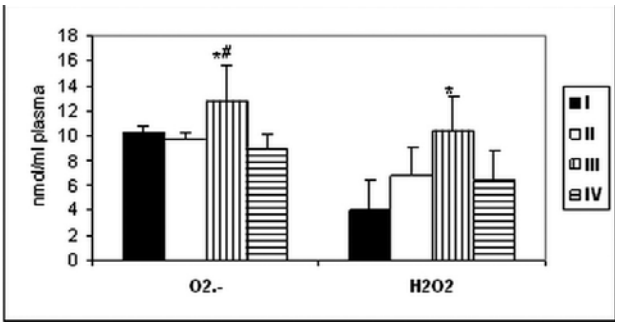

Fig. 1. The plasma ROS concentrations in control (I and II), PHZ-treated (III) and bled rats (IV). Values represent means ± SEM for 5 animals per group. *p < 0.05, compared to the control group (I), #p<0.05, PHZ-treated versus bled rats

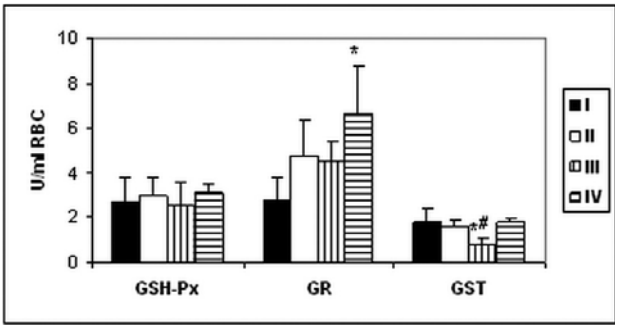

Fig. 2. The activities of lysate glutathione peroxidase (GSH-Px), glutathione reductase (GR) and glutathione-S-transferase (GST) in control (I and II), PHZ-treated (III) and bled rats (IV). Values represent mean ± SEM for 5 animals per each group. Values for GSH-Px are in U/ml RBC x 10^4, and for GR in U/ml RBC x 10^5 and GST are in U/ml RBC x 10^3. *p < 0.05, compared with the control group (I), #p < 0.05, PHZ-treated versus bled rats

The TBARS levels in the RBC of all investigated groups of animals were not changed. The RBC concentration of GSH was significantly higher, while the GSSG level and GSSG/2 GSH ratio were significantly lower in both anemic groups in comparison with the control group (I) (Table. 2). When the two groups of anemic rats were compared, the level of GSH was significantly lower in the PHZ (III) vs. bled rats (IV).

The GSH-Px activity was not different in the investigated groups of rats (Fig. 2). The activity of GR was significantly higher in the bled rats compared to the control group (Fig. 2). The activity

of GST was significantly lower in the PHZ-treated rats compared to the control group. When two groups of anemic rats were compared, the activity of the GST was significantly lower in the PHZ (III) vs. the bled rats (IV) (Fig. 2).

DISCUSSION

In experimental conditions reticulocytosis can be induced by daily bleeding or PHZ treatment (Kostić et al., 1990; Marković et al., 2006; 2007; 2009). Recent studies have showed that PHZ-induced reticulocytes are a simple model system for investigating anemia and apoptosis (Diwan et al. 2008; Ramot et al. 2008; Savill et al. 2009).

Our results showed no significant alterations in the levels of the ROS in the plasma of the bled rats, but showed a significantly high level of TBARS. The elevated level of lipid peroxidation in the bled rats may be the consequence of hemorrhagic shock which results in oxidative stress and is followed by significant elevations in TBARS (Mauriz et al., 2001).

In the plasma and RBC of the bled animals the concentration of GSH is significantly higher, while the concentration of GSSG and the ratio GSSG/2 GSH, are significantly lower in this group of anemic animals. All these changes and the efficacy of the glutathione status are a consequence of the higher activity of GR in the bled rats. Glutathione reductase catalyzes the reduction of oxidized GSH back into GSH, the latter being the co-substrate of GSH-Px (Gul et al., 2000). Higher GR activity provides conservation of GSH which is a primary protective molecule from the oxidative stress of the RBC. Based on the presented results, we can conclude that the glutathione system is efficient in the antioxidative defense of RBC of bleeding-induced anemic animals, but less efficient in the plasma of the bled animals considering the high levels of the TBARS.

According to our results, PHZ treatment was followed by an accumulation of $O_2^{\cdot-}$ and H_2O_2 in the plasma. On the other hand, there was no change in lipid peroxidation in either the plasma, or the RBC of the PHZ-treated animals. The high levels of ROS cause the elevation of Heinz body formation in the RBC of the PHZ-treated rats (Marković et al., 2009), indicating that proteins (e.g. hemoglobin) could be the main site of PHZ-induced damage of RBC (McMillan et al., 2005).

The presented results also showed that the GSSG/2GSH ratio is significantly higher in the plasma of PHZ-treated rats, which indicates an inefficient metabolism of the glutathione system and could be one of the causes of ROS accumulation. We can also conclude that the plasma of the PHZ-treated rats is the main site of oxidative stress mainly because the glutathione antioxidative mechanisms are less efficient in the scavenging of ROS, but there is good protection of this mechanism against lipid peroxidation.

In the RBC of PHZ-treated rats, the GSSG/2GSH ratio is significantly lower compared to the control group. In this condition, GSH probably reacts rapidly as a scavenger of some of the ROS (Kalyanaraman et al., 1996), and directly or indirectly reduces semidehydroascorbate and in that way stops the process of lipid peroxidation (Chan et al., 1999). These reactions, directly or indirectly, result in the production of GSSG which is then reduced intracellularly to GSH with GR through a NADPH-dependent reaction (Mates, 2000).,GSH protected the RBC of anemic rats from oxidative damage, mainly by stopping the chain reaction of peroxidation, and this antioxidative system was more inefficient in the protection of hemoglobin oxidation (Marković et al., 2009).

In the RBC of PHZ-treated animals the activity of GST is significantly lower compared to the control group, as well as compared to the group treated by daily bleeding. The role of GST is seen through detoxification, transport and synthesis (Bolt, 1996), but it is also one of the oxidative stress-inducible enzymes. The high level of peroxynitrites in the RBC of PHZ- treated animals (Markovic et al., unpublished data) may be the cause of the halting of GST activity (Wong et al, 2001).

When the two groups of anemic animals are compared, there is an accumulation of ROS and a less effective glutathione antioxidative system in the plasma of the PHZ-treated rats, as well as hemorrhagic shock-induced lipid peroxidation in the plasma of the bled rats. All these data indicate higher oxidative stress in the plasma and RBC of the PHZ-treated group compared to the group of bled rats.

Previous data showed that PHZ-induced reticulocytes maturated into erythrocytes normally *in vitro* (Gronowicz et al., 1984), as well *in vivo* (Kostić et al., 1990). In addition, our recent studies showed that PHZ-induced reticulocytes are an experimental system for oxidative stress. However, when compared with untreated erythrocytes, reticulocytes represent a system adapted to oxidative stress (Marković et al., 2006, 2007). Taking all this into account, this study contributes to a better definition of the metabolic processes in PHZ-induced reticulocytes, as simple model system for investigation of anemia, apoptosis and mitochondria-based processes.

Acknowledgment - This work was supported by the Ministry for Science and Technological Development of the Republic Serbia, Grant No 143035B.

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ГЛУТАТИОНСКИ СТАТУС У КРВИ ПАЦОВА НАКОН РЕТИКУЛОЦИТОЗЕ ИЗАЗВАНЕ ФЕНИЛХИДРАЗИНОМ И КРВАРЕЊЕМ

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Циљ овог рада је био да се испрате *in vivo* ефекти фенилхидразином (PHZ) и крварењем индуковане ретикулоцитозе на параметре редокс и глутатионског антиоксидативног статуса у плазми и црвеним крвним ћелијама (RBC) пацова. Резултати показују нижи ниво реактивних врста кисеоника (ROS), виши ниво липидне пероксидације и ефикасну антиоксидациону улогу глутатионског система у крви исквављених пацова. Третман PHZом проузроковао је више концентрације ROSa и акумулацију оксидованог глутатиона у плазми, док је глутатионски систем показао ефикасан антиоксидативни капацитет у RBCу пацова. Када се упореде две анемичне групе, израженији оксидациони стрес се јавља у плазми пацова третираних PHZом.