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Short communication

THE EFFECTS OF SUPEROXIDE DISMUTASE KNOCKOUT ON THE OXIDATIVE STRESS PARAMETERS AND SURVIVAL OF MOUSE ERYTHROCYTES

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Abstract: The erythrocytes of 12-month old *Sod1*$^{-/-}$ mice showed an increased level of reactive oxygen species (ROS), as estimated by the degree of dihydroethidine and dihydrorhodamine oxidation, and the increased level of Heinz bodies. No indices of severe oxidative stress were found in the red blood cells and blood plasma of *Sod1*$^{-/-}$ mice as judged from the lack of significant changes in the levels of erythrocyte and plasma glutathione, plasma protein thiol and carbonyl groups and thiobarbituric-acid reactive substances in the blood plasma. However, a decreased erythrocyte lifespan, increased reticulocyte count and splenomegaly were noted, indicating the importance of superoxide dismutase for maintaining erythrocyte viability. The levels of erythrocyte ROS

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Abbreviations used: DMSO – dimethylsulfoxide; DT – dithionite; DTPA – diethylenetriaminepentaacetatic acid; EDTA – ethylenediaminetetraacetic acid; GPx – glutathione peroxidase; GSH – glutathione; GSSG – glutathione disulfide; Hb – hemoglobin; HB – Heinz bodies; HBS – Hank's buffered solution; Ig – immunoglobulin; KO – knockout; NEM – N-ethylmaleimide; NHS - N-hydroxysuccinimide; PE – phycoerythrin; Prdx – peroxiredoxin; RBC – red blood cell; SOD – superoxide dismutase; TBARS – thiobarbituric-acid reactive substances; tBOOH – tert-butyl hydroperoxide

and Heinz bodies and the reticulocyte count were indistinguishable in *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ mice, suggesting that a superoxide dismutase activity decrease to half of its normal value may be sufficient to secure the protective effects of the enzyme.

Key words: Superoxide dismutase, Erythrocyte, Red blood cell, Reactive oxygen species, Oxidative stress, Heinz bodies, Acetylcholinesterase

INTRODUCTION

The mammalian erythrocyte functions as an oxygen transporter, and is thus permanently in danger of oxidative damage. In order to avert this danger, the erythrocyte is well equipped with antioxidants and antioxidative enzymes. The relative importance of the various components of the red blood cell antioxidant barrier is an intriguing issue worthy of study.

The role of the regeneration of glutathione in the protection of erythrocytes against redox-cycling xenobiotics generating oxidative stress is evident in favism. In this case, an inborn deficiency of glucose 6-phosphate dehydrogenase activity leads to erythrocyte destruction upon exposure to fava beans or certain drugs [1, 2]. By contrast, no significant anomalies in physiological erythrocyte functions have been reported in acatalasemia [3, 4] and glutathione reductase deficiency [4]. Studies of knockout mice provide unique information on the importance of various antioxidant proteins in erythrocyte physiology. Such studies have demonstrated that Prdx1-deficient mice showed decreased erythrocyte survival and hemolytic anemia [5], indicating a significant physiological role of peroxiredoxin 1 in the erythrocyte. Superoxide dismutase (CuZnSOD, SOD1) is another fundamental antioxidant enzyme of the red blood cell, and is the only form of SOD present in mature mammalian erythrocytes. It has been found that *Sod1*$^{-/-}$ mice have a reduced lifespan with indices of oxidative stress in their blood such as increased serum iron and decreased plasma ascorbate [6-8]. Their erythrocytes show a decreased survival rate, increased vulnerability to oxidative stress and augmented binding of autologous IgG [9]. Studies of the effects of SOD1 knockout have been done on relatively young, mostly 3-month old animals. The aim of this study was to examine erythrocyte survival and selected oxidative stress parameters of the erythrocytes and blood plasma of 12-month old mice deficient in SOD1.

MATERIALS AND METHODS

Animals

Mice homozygous for the non-functional SOD1 allele (KO SOD1), producing no SOD1 protein, and control mice homozygous for the wild-type SOD1 allele (wt) were used in the experiments. The mice in both groups were 12 months old. A breeder pair of mice (strain B6; 129S7-Sod1$^{\mathbf{tm1Leb}}$) heterozygous for a SOD1$^{\mathbf{tm1Leb}}$-targeted mutation [10] and their progeny were provided by the

Jackson Laboratory (Bar Harbor, ME). Males and females heterozygous for the non-functional SOD1 allele ($SOD1^{-/+}$) were intercrossed, and their progeny were kept at 24-25ºC, in 80% humidity with a light-dark cycle of 12 h. The mice were fed a standard laboratory diet (Labo-feed, Kcynia, Poland) and water *ad libitum*. Genotyping using DNA isolated from the mouse tails was performed by PCR analysis according to a protocol provided by the Jackson Laboratory. All the experimental procedures involving animals were approved by the 3rd Local Ethical Commission (permission number 46/2006).

Hematological analysis
Blood was drawn from the mice by direct cardiac puncture immediately after death, and was treated with heparin as an anticoagulant. The erythrocyte counts and cell parameters were determined using an automated Sysmex F-820 Analyzer.

Red blood cell turnover assay
The red blood cell lifespan was assayed by *in vivo* biotinylation followed by fluorescence-activated cell sorting (FACS) analysis. RBCs were labeled with biotin by intravenous injection of 100 μL of a 30 mg/mL solution of (+)-Biotin N-hydroxysuccinimide ester (Sigma) dissolved in 10% DMSO (Sigma) and diluted with 0.9% NaCl (pH 7.4). The first blood sample used to determine the quantity of biotinylated cells was taken 60 min after the injection of the biotin derivative. More than 96% of the murine RBCs were found to be biotinylated. Thereafter, blood samples were collected from the tail vein at various intervals for 4 weeks to quantify the number of biotin-labeled cells remaining in the circulatory system. Sampled cells were washed with HBS (10 mM HEPES, 165 mM NaCl, pH 7.4) and incubated with streptavidin-phycoerythrin conjugate (Invitrogen; 1 μg/ml in the same buffer) for 30 min at room temperature in the dark. Unbound streptavidin was removed by washing with HBS, and PE-labeled cells were then detected by flow cytometry (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ). Counts of positive (PE-labeled) cells compared with the total number of RBCs were used to calculate the percentage of biotinylated cells.

Measurement of the intracellular reactive oxygen species contents
The intracellular peroxide and superoxide levels were estimated by respectively labeling cells with dihydrorhodamine 123 and dihydroethidine. The fluorescent probes were obtained from Invitrogen (Karlsruhe, Germany). Blood was diluted 100-fold with PBS, the appropriate probe was added to a final concentration of 5 μM, and the suspensions were incubated at 37ºC in the dark for 1 h. A total of 10^5 cells were then counted with an LSRII multicolor flow cytometer (Becton Dickinson).

Heinz body and reticulocyte counts
Heinz bodies (HB) and reticulocyte numbers were examined in blood smears stained with Brilliant Cresyl Blue (BCB, Merck). Equal volumes (usually 20 μl)

of blood and 1% BCB solution were mixed in a microfuge tube and incubated in the dark at room temperature for 30 min. 10 µl of the stained suspension was then smeared onto a microscope slide and air-dried. The slides were inspected at 1,000 × magnification. To determine the percentage of HB-containing cells or reticulocytes, a total of 1,000 red blood cells were analyzed on each slide.

Evaluation of the osmotic fragility

Osmotic fragility was determined by incubating whole blood aliquots in 20 volumes of NaCl solutions (at a concentration between 120 and 300 mM) buffered with 1 mM phosphate buffer, pH 7.4, or in 1 mM phosphate buffer, pH 7.4 (the control for 100% hemolysis). The samples were then centrifuged (1,000 g × 10 minutes) and the absorbance of the supernatant was measured at 540 nm. The 50% hemolysis concentration was calculated after logarithmic linearization of an osmotic fragility curve for the concentration range 160-240 mM NaCl according to Judkiewicz *et al.* [11].

Determination of the superoxide dismutase activity

Superoxide dismutase (SOD) activity was estimated on the basis of the inhibition of adrenaline autoxidation [12]. Briefly, an erythrocyte hemolysate was subjected to the Tsuchihashi treatment to remove hemoglobin, and then added to a 333-µM solution of adrenalin in 50 mM carbonate, pH 10.2, containing 100 µM EDTA. The absorbance of the solution was monitored at 480 nm as a function of time at 37ºC. The degree of inhibition of the maximal rate of absorbance increase was a measure of SOD activity. A unit of SOD activity was defined as the activity which decreases the maximal rate of absorbance increase (0.025 min^{-1} in the control) by 50% [13]. SOD activity was expressed per milligram of hemoglobin.

Determination of the catalase activity

The activity of catalase in the erythrocyte lysate was assayed by monitoring the removal of exogenously added hydrogen peroxide [14]. Briefly, murine erythrocytes were lysed in water and the lysate (at a final concentration corresponding to a hematocrit of 0.125%) was incubated for 3.5 minutes in a reaction mixture containing 50 mM hydrogen peroxide. Subsequently, the catalase reaction was stopped by adding 15 mM sodium azide, and the remaining hydrogen peroxide was assayed in a chromogenic reaction with 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid, catalysed by horseradish peroxidase. The catalase activity was expressed per milligram of hemoglobin.

Determination of the glutathione peroxidase activity

The activity of glutathione peroxidase in the erythrocyte lysate was assayed by the indirect method with glutathione reductase using *t*-butyl hydroperoxide (tBOOH) as a substrate [15]. Briefly, murine erythrocytes were lysed with water and the lysate (at a final concentration corresponding to a hematocrit of 0.8%)

was incubated in a mixture containing 0.25 mM NADPH, 2 mM GSH, 0.5 U/ml glutathione reductase and 1.2 mM tBOOH. The decrease in NADPH concentration was measured by monitoring the decrease in absorbance at 340 nm using a Victor2 (Wallac) microplate reader. The GPx activity was expressed per milligram of haemoglobin.

Determination of the acetylcholinesterase activity

Acetylcholinesterase activity was determined in red blood cell membranes isolated by hypotonic lysis. Briefly, 1 volume of packed cells, after the plasma was washed off, was diluted with 19 volumes of 1 mM phosphate buffer pH 7.4, incubated on ice for 20 min and centrifuged at 12,000×g for 15 min. The remaining hemoglobin was removed in two subsequent washing steps. The protein content in the membrane pellet was determined by the method of Lowry *et al.* [16] using bovine serum albumin as a standard. The enzyme activity was determined by method of Ellman *et al.* with minor modifications [17].

Determination of the concentration of free thiol groups in the plasma

The concentration of free thiol groups in the plasma was assayed by the monobromobimane method [18]. Briefly, plasma samples were diluted 2x in phosphate-buffered saline, monobromobimane (a thiol-specific reagent which generates a fluorescent product upon reaction with thiol groups) was added to a final concentration of 100 μM, and the initial rate of reaction was measured by following the rate of increase in fluorescence (excitation wavelength 380 nm, emission wavelength 490 nm) using a Fluoroskan Ascent FL (Thermo Labsystems) kinetic fluorimeter. A calibration curve was prepared using reduced glutathione as standard and the results were expressed as thiol group concentration.

Determination of the concentration of reduced and oxidized glutathione in the plasma and erythrocyte lysate

The concentrations of GSH and GSSG in the plasma and erythrocyte lysate were assayed by the *o*-phthalaldehyde method [19]. Briefly, plasma or erythrocyte lysate samples were redox-quenched and deproteinized in a solution containing ascorbic acid, DTPA and trichloroacetic acid. The acid-soluble extract was neutralised and an aliquot was reacted with *N*-ethylmaleimide (NEM) in order to block the free thiol group of reduced glutathione. Subsequently, an aliquot of the NEM-reacted sample was reacted with sodium dithionite to reduce any GSSG present. Finally, all the samples were reacted with *o*-phthalaldehyde and the fluorescence (excitation wavelength 355 nm, emission wavelength 430 nm) was measured using a Fluoroskan Ascent FL (Thermo Labsystems) fluorimeter. Authentic GSH and GSSG were used to prepare a calibration curve. The GSH concentration was derived from the difference between the unreacted and NEM-reacted sample, while the GSSG concentration was derived from the difference between the DT-reacted and NEM-reacted sample.

Estimation of the lipid peroxidation product levels
Lipid peroxidation was estimated by measuring the absorbance of thiobarbituric acid-reactive materials at 532 nm [20].

RESULTS

The level of SOD activity, estimated by inhibiting adrenalin autoxidation, was considerably decreased in the erythrocytes of the *Sod1*$^{+/-}$ mice and further decreased in the erythrocytes of the *Sod1*$^{-/-}$ mice (Tab. 1). The level was not zero in the red blood cells of the *Sod1*$^{-/-}$ mice, due to the interference of low-molecular antioxidants known to occur in the adrenaline method of determining SOD activity [12]. The absence of SOD activity in the erythrocytes of *Sod1*$^{-/-}$ mice was verified by gel electrophoresis followed by activity staining and the determination of enzymatic activity (not shown). The erythrocytes of the *Sod1*$^{-/-}$ mice showed an increased level of superoxide as detected by the oxidation of dihydroethidine, a superoxide-specific fluorogenic probe. The increased level of superoxide was accompanied by an increased level of reactive oxygen species able to oxidize dihydrorhodamine, apparently mostly H_2O_2, which can oxidize this probe in the erythrocyte due to the pseudoperoxidase activity of hemoglobin [21, 22] (Fig. 1). The level of Heinz bodies, thought to be due to oxidative stress in the erythrocyte, increased in the red blood cells of the *Sod1*$^{-/-}$ mice (Fig. 2).

Tab. 1. Selected parameters of the erythrocytes and of the blood plasma redox status in the control and SOD1 knockout mice. Number of animals studies are given in parenthesis.

Mice genotype	*Sod1*$^{+/+}$	*Sod1*$^{+/-}$	*Sod1*$^{-/-}$
Erythrocyte SOD (units/g Hb)	5.0 ± 3.0 (6)	11.2 ± 3.6* (5)	17.3 ± 4.4* (10)
Erythrocyte GSH (μmole/g Hb)	6.1 ± 0.7 (6)	5.9 ± 1.0 (5)	6.1 ± 1.3 (10)
Erythrocyte GSSG (μmole/g Hb)	1.1 ± 0.1 (6)	1.0 ± 0.1 (5)	1.3 ± 0.3 (10)
Plasma GSH (μM)	3.2 ± 1.6 (6)	2.0 ± 0.3 (5)	3.1 ± 2.9 (10)
Plasma GSSG (μM)	89.1 ± 12.3 (6)	87.4 ± 17.7 (5)	98.0 ± 7.9 (10)
Plasma –SH (μmole/g protein)	6.0 ± 1.4 (5)	6.0 ± 1.5 (5)	5.4 ± 1.0 (10)
Plasma protein carbonyls (μmole/g protein)	0.67 ± 0.28 (5)	0.96 ± 0.27 (5)	0.88 ± 0.27 (5)
Erythrocyte catalase (U/mg Hb)	32.9 ± 3.3 (10)		40.7 ± 9.6 (10)
Erythrocyte glutathione peroxidase (U/g Hb)	0.44 ± 0.19 (10)		0.49 ± 0.15 (10)
Erythrocyte TBARS (A_{576}/g Hb)	0.32 ± 0.13		0.29 ± 0.12
Acetylcholinesterase activity (U/mg protein)	64.1 ± 20.7 (10)		100.4 ± 40.4 (10)*
Osmotic fragility of erythrocytes (c_{50}) (mM NaCl)	206 ± 3 (10)		202 ± 7 (10)

*P < 0.02 with respect to *Sod1*$^{+/+}$ mice

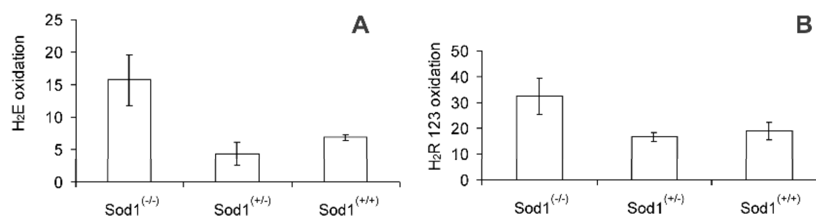

Fig. 1. The levels of reactive oxygen species in the erythrocytes of the *Sod1*$^{+/+}$, *Sod1*$^{+/-}$ and *Sod1*$^{-/-}$ mice. The levels of superoxide and peroxides were respectively estimated by flow cytometric evaluation of the oxidation of A – dihydroethidine (H_2E) and B – dihydrorhodamine 123 (H_2R123). Relative fluorescence is shown on the axis of ordinates. *Sod1*$^{-/-}$ mice vs. *Sod1*$^{+/-}$ and *Sod1*$^{-/-}$ mice: $P < 0.05$.

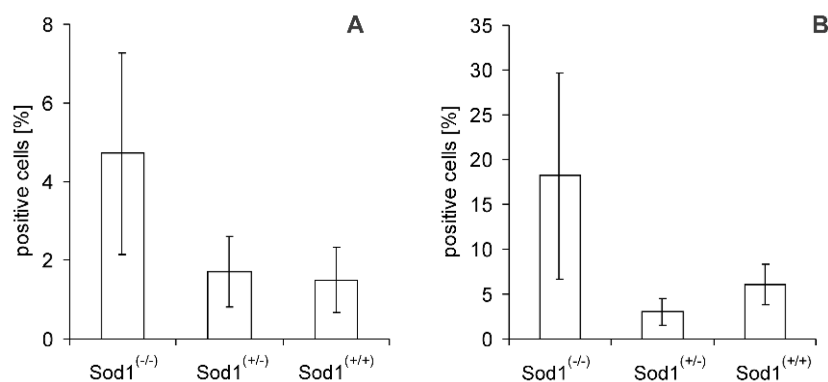

Fig. 2. The reticulocyte count (A) and level of Heinz bodies (B) in the erythrocytes of the *Sod1*$^{+/+}$, *Sod1*$^{+/-}$ and *Sod1*$^{-/-}$ mice expressed as a percentage of positively staining cells. *Sod1*$^{-/-}$ mice vs. *Sod1*$^{+/-}$ and *Sod1*$^{-/-}$ mice: $P < 0.05$.

The oxidative stress due to the increased level of reactive oxygen species (ROS) in the erythrocytes did not lead to drastic changes in the redox state of the erythrocytes and blood plasma. The contents of GSH and GSSG (Tab. 1), and the level of methemoglobin were unchanged in the *Sod1*$^{+/-}$ and *Sod1*$^{-/-}$ mice. The activities of main antioxidant enzymes, catalase and glutatione peroxidase, were not altered; however, the activity of acetylcholinesterase was significantly increased in the erythrocytes of the *Sod1*$^{-/-}$ mice.

The blood plasma indices of oxidative stress were not altered in the *Sod1*$^{-/-}$ mice. The level of protein thiols, protein carbonyls and thiobarbituric-acid reactive products of lipid peroxidation were not different from those of wild-type mice (Tab. 1).

Previous hematological comparisons showed a decrease in the reticulocyte count, hematocrit and hemoglobin level, with a concomitant increase in the erythrocyte mean volume [23]. In this study, we found a significant shortening of the survival of erythrocytes in the *Sod1*$^{-/-}$ mice (Fig. 3). Splenomegaly was also observed in association with this effect (Fig. 4).

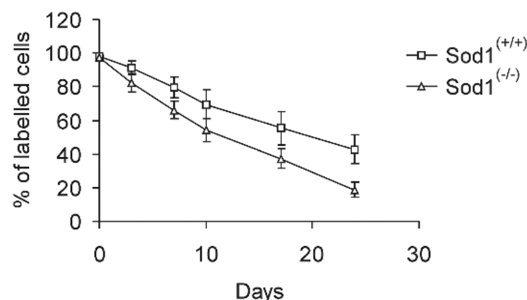

Fig. 3. The shortened lifespan of *Sod1*$^{-/-}$ erythrocytes. Erythrocyte survival was determined in mice injected intravenously with NHS-biotin. 5 ml of blood was drawn from the tail vein at the indicated time points, and reacted with R-phycoerythrin-conjugated streptavidin followed by flow cytometry analysis. The percentage of biotinylated erythrocytes was calculated as a ratio of positive cells to all erythrocytes. Six mice of each genotype were used. $P < 0.05$ starting from 7 days.

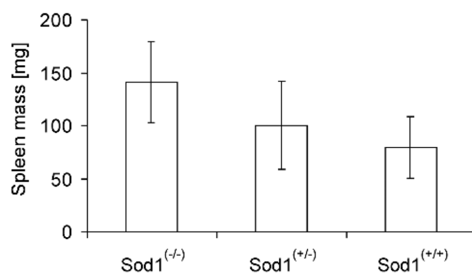

Fig. 4. The spleen mass of the *Sod1*$^{+/+}$, *Sod1*$^{+/-}$ and *Sod1*$^{-/-}$ mice. *Sod1*$^{-/-}$ mice vs. *Sod1*$^{-/-}$ mice: $P < 0.05$.

DISCUSSION

Superoxide dismutase is an enzyme present ubiquitously in aerobic cells, and a lack of CuZnSOD (SOD1), responsible for the vast majority of intracellular SOD activity, can be expected to significantly affect the physiology of eukaryotic cells and organisms. Although, somewhat surprisingly, *Sod1*$^{-/-}$ mice develop normally, the females show reduced fertility, have a shortened lifespan, and have increased hepatocarcinogenesis, increased levels of 8-oxo-deoxyguanosine and F2-isoprostanes, a reduction in plasma ascorbate and age-dependent skeletal muscle atrophy [6-8].

SOD1 is the only form of superoxide dismutase present in mature erythrocytes, which are devoid of mitochondria. Therefore, one may expect that a deficiency or complete lack of CuZnSOD would have significant consequences, especially for red blood cells. Previous studies revealed changes in the hematological parameters of *Sod1*$^{-/-}$ mice, including a lower erythrocyte count, hematocrit and

hemoglobin content and an increased size of erythrocytes [23]. The study by Iuchi *et al.* showed an increased level of reactive oxygen species in the erythrocytes, an increased level of lipid peroxidation products, increased hemoglobin susceptibility to oxidation, decreased glutathione peroxidase activity, a shortened lifespan of SOD1-deficient erythrocytes, and splenomegaly in *Sod1*$^{-/-}$ mice [9].

In this study, performed on 12-month old mice, we confirmed the increased level of reactive oxygen species in the erythrocytes of *Sod1*$^{-/-}$ mice with two fluorogenic probes, dihydroethidine (specific for superoxide) and dihydrorhodamine 123 (specific for peroxide-derived oxidants) [24, 25]. We did not find many other indices of generalized oxidative stress in the erythrocytes and blood plasma. The level of reduced and oxidized glutathione was unchanged in the erythrocytes of the SOD1 knockout mice, which concurs with the data of Iuchi *et al.* [9]. These results should be treated with care because, for logistical reasons, the glutathione analysis could only be done several hours after taking the blood, which resulted in artifactually high levels of reduced glutathione; in any case, they demonstrate no increased tendency towards oxidation of erythrocyte glutathione in erythrocytes devoid of SOD1. Although there was a tendency towards an increase in the blood plasma level of reduced glutathione, a lowered content of plasma protein thiol groups and an augmented level of plasma protein carbonyls, the differences in the values of these parameters in the *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice did not reach the level of statistical significance. These results indicate a limited extent of oxidative stress in the blood of *Sod1*$^{-/-}$ mice, in spite of the oxidative stress occurring intracellularly, as found for erythrocytes [9], liver [6] and muscle [7, 26].

However, the level of Heinz bodies, products of oxidative denaturation of hemoglobin, was considerably augmented in erythrocytes devoid of SOD1, demonstrating the enhanced oxidative damage to red blood cell proteins in the absence of superoxide dismutase. We also found a decreased lifespan of erythrocytes of *Sod1*$^{-/-}$ mice with a concomitant increase in the level of reticulocytes, apparently released in higher amounts to the circulation to compensate the enhanced erythrocyte loss, and splenomegaly, resulting from accelerated destruction of the erythrocytes. Therefore, local oxidative stress due to the absence of superoxide dismutase shortens the lifespan of mouse erythrocytes. Iuchi *et al.* demonstrated increased binding of autologous IgG to the erythrocytes of *Sod1*$^{-/-}$ mice [9], which may mediate their accelerated elimination [27, 28]. The results obtained by us for 12-month old *Sod1*$^{-/-}$ mice are therefore in general agreement with the findings by Iuchi *et al.* for 14-week and 40-week old mice [9].

We did not observe significant differences in the activities of catalase and glutathione peroxidase between the erythrocytes of *Sod1*$^{+/+}$ mice and *Sod1*$^{-/-}$ mice. The data of Iuchi *et al.* indicates decreased glutathione peroxidase activity and a lack of changes in catalase activity [9]. We found an increased activity of acetylcholinesterase in the erythrocytes of *Sod1*$^{-/-}$ mice. Acetylcholinesterase

activity decreases during erythrocyte aging and has been suggested to be a marker of red cell age [29, 30]. The elevated activity of this enzyme in the erythrocytes of *Sod1*$^{-/-}$ mice may reflect their accelerated removal and resulting lower mean age. This enzyme, located at the outer surface of the erythrocyte membrane, is not subject to intracellular oxidative stress in erythrocytes devoid of superoxide dismutase, in contrast to catalase and glutathione peroxidase, which are present inside the cell and subject to an increased steady-state level of superoxide. Both enzymes are known to be inactivated by superoxide [13], which apparently explains the lack of increase in their activities, which could be anticipated on the basis of the lower mean age of the circulating erythrocytes (the activity of these enzymes also decreases during erythrocyte aging, as in the case of acetylcholinesterase) [30, 31].
Although we were not able to analyze all the parameters in the *Sod*$^{+-/-}$ heterozygotes, we compared the levels of reactive oxygen species and the reticulocyte and Heinz body counts, and also assessed splenomegaly in order to address the question of whether a decrease of SOD activity to half of the normal value yields a gene dose effect or the remaining enzyme activity in the erythrocytes of *Sod*$^{+-/-}$ mice is sufficient to afford efficient protection against superoxide. While the results concerning the mass of the spleen are inconclusive, those concerning the level of reactive oxygen species evidence that the remaining activity of superoxide dismutase is sufficient to control the production of ROS measured with fluorogenic probes at a level not different from that typical for the erythrocytes of the wild-type mice. Similarly, the level of Heinz bodies and the reticulocyte count did not differ between the erythrocytes of the *Sod1*$^{+/+}$ and *Sod1*$^{+/-}$ mice. It seems therefore that half of the normal level of erythrocyte SOD may be sufficient to secure at least some of the intracellular biological functions of this enzyme. This conclusion may be relevant, for example, for the interpretation of the results obtained for *Sod1*$^{+/-}$ mice, including the lack of significant lifespan shortening of the heterozygotes, in contrast to *Sod1*$^{-/-}$ mice [6].

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