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Haemolytic anaemia and alterations in hepatic iron metabolism in aged mice lacking Cu,Zn-superoxide dismutase

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The continuous recycling of haem iron following phagocytosis and catabolism of senescent and damaged red blood cells by macrophages is a crucial process in the maintenance of systemic iron homoeostasis. However, little is known about macrophage iron handling in haemolytic states resulting from a deficiency in antioxidant defences. Our observations indicate that the recently described chronic, but moderate regenerative, haemolytic anaemia of aged SOD1 (superoxide dismutase 1)-knockout mice is associated with red blood cell modifications and sensitivity to both intra- and extra-vascular haemolysis. In the present study, we have characterized the molecular pathways of iron turnover in the liver of *Sod1*-deficient mice. Despite iron accumulation in liver macrophages, namely Kupffer cells, we did not measure any significant change in non-haem liver iron. Interestingly, in Kupffer cells, expression of the rate-limiting enzyme in haem degradation, haem oxygenase-1, and expression of the iron exporter ferroportin were both up-regulated, whereas the hepcidin mRNA level in the liver was decreased in $Sod1^{-/-}$ mice. These results suggest that concerted changes in the hepatic expression of iron- and haem-related genes in response to haemolytic anaemia in $Sod1^{-/-}$ mice act to reduce toxic iron accumulation in the liver and respond to the needs of erythropoiesis.

Key words: erythrophagocytosis, haemolysis, haem oxygenase, iron, Kupffer cells, superoxide dismutase 1.

INTRODUCTION

ROS (reactive oxygen species) are by-products of normal aerobic metabolism. Several antioxidant systems exist in cells to provide protection against ROS toxicity. The principal defence against the toxicity of superoxide radical ($O_2^{\bullet -}$), released mainly into the cytosol [1], is SOD1 (superoxide dismutase 1; Cu,Zn-SOD). Surprisingly, despite this crucial protective role, homozygous mutant mice lacking *Sod1* are viable and phenotypically normal [2–4].

Permanent oxidative stress occurring due to elevated ROS later in life is an important mechanism in the pathogenesis of aging [5]. In older mice, SOD1 deficiency results in an increased incidence of pathological changes, such as hepatocarcinogenesis, hearing loss and muscle atrophy [6–8]. As a consequence, the lifespan of *Sod1*-knockout mice is reduced by approx. 30 % [3,4].

Mice with genetic *Sod1* deficiency have been extensively used as an experimental animal model to explore SOD1 dysfunction-related pathologies [6,8,9]. With regard to haematological disorders, the critical role of alterations in the redox state due to SOD1 deficiency has been reported in HA (haemolytic anaemia) occurring in $Sod1^{-/-}$ mice [10], as well as in the impairment of haematopoietic cell development in the mouse model of Fanconi anaemia [11].

In the present study we examined the consequences of HA in 1-year-old mice lacking Cu,Zn-SOD1 activity, focusing on alterations in their hepatic iron metabolism. Our results clearly indicate that the enhanced destruction and accelerated removal of RBCs (red blood cells) from the circulation of aged $Sod1^{-/-}$ mice induces substantial changes in the expression of genes involved in haem and iron homoeostasis. The results of the present study establish a new link between oxidative stress due to the combined effects of SOD1 deficiency and age-dependent changes in redox state and the regulation of iron metabolism at the systemic level.

EXPERIMENTAL

Animals

Mice homozygous for the non-functional *Sod1* allele, and control mice homozygous for the wt (wild-type) *Sod1* allele were used in experiments at the age of 1 year. A breeder pair of mice (strain B6;129S7-$SOD1^{tm1Leb}$) heterozygous for a $Sod1^{tm1Leb}$-targeted mutation [3] were provided by The Jackson Laboratory. The Third Local Ethical Commission approved all experimental procedures involving animals.

Analysis of RBC and serum iron parameters

Blood was drawn from mice by direct cardiac puncture immediately after death and was treated with heparin. RBC and reticulocyte counts, and cell parameters, as well as serum iron, were determined using an automated Sysmex F-820 Analyser. Serum transferrin was measured at the Laboratory of Biochemistry at the Institut Federatif de Recherche 02, CHU Bichat-Claude Bernard (Paris, France).

Real-time quantitative RT (reverse transcriptase)–PCR

Hepatic Fpn (ferroportin), Hepc (hepcidin), CD91 and CD163 mRNA levels were measured by a real-time quantitative PCR

Abbreviations used: BMDM, bone marrow-derived macrophage; ECL, enhanced chemiluminescence; EP, erythrophagocytosis; Fpn, ferroportin; Ft, ferritin; HA, haemolytic anaemia; Hepc, hepcidin; H-Ft, heavy-Ft; HO-1, haem oxygenase-1; Hp, haptoglobin; Hx, haemopexin; KC, Kupffer cell; L-Ft, light-Ft; PS, phosphatidylserine; RBC, red blood cell; ROS, reactive oxygen species; RT, reverse transcriptase; SOD1, superoxide dismutase 1; wt, wild-type.

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method as described previously [12]. Real-time quantitative RT–PCR of cDNAs derived from specific transcripts was performed in a Light Cycler (Roche Diagnostics) using the pairs of oligonucleotide primers indicated in Supplementary Table S1 (at http://www.BiochemJ.org/bj/420/bj4200383add.htm). For data analysis, Light Cycler 3.5 Software was used. Expression was quantified relative to that of a control transcript encoding GAPDH (glyceraldehyde-3-phosphate dehydrogenase).

Western blot analysis

For Western blot analysis, 50 μg of liver cytosolic extracts [for Ft (ferritin) subunit detection] or 100 μg of liver membrane extracts [for HO-1 (haem oxygenase-1) and Fpn detection] were resolved on SDS/PAGE (14 % and 10 % gels respectively). To determine plasma Hp (haptoglobin) and Hx (haemopexin) levels, samples were separated on SDS/PAGE (10 % gels). Resolved proteins were electroblotted on to Hybond-ECL (where ECL is enhanced chemiluminescence) nitrocellulose membranes (Amersham Life Sciences). The membranes were initially blocked by gentle agitation in TBST [Tris buffered saline (50 mM Tris/HCl, pH 7.6, and 150 mM NaCl) containing 0.15 % Tween 20] containing 5 % fat-free skimmed milk powder for 1 h at room temperature (20 °C) followed by overnight incubation at 4 °C with the following primary antibodies: rabbit antisera raised against recombinant mouse H- and L-Ft (heavy- and light-Ft repectively; kindly provided by Dr P. Santambrogio, Department of Bio Technology, San Raffaele Scientific Institute, Milan, Italy), a rabbit polyclonal antibody raised against rat liver HO-1 (StressGen), a rabbit antibody raised against mouse Fpn [13], a chicken antibody raised against human Hp (USBiological), and a goat antiserum raised against human Hx (kindly provided by Dr E. Tolosano, Molecular Biotechnology Center, University of Torino, Torino, Italy). Membranes were then washed and incubated with peroxidase-conjugated anti-rabbit, anti-chicken or anti-goat secondary antibodies (Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactive bands were detected using the ECL Plus Western blotting detection system (Amersham Life Sciences). Quantification was performed relative to actin detected using a specific antibody (Santa Cruz Biotechnology).

Immunohistofluorescence

Mouse livers were fixed in Bouin's fixative for 72 h at 20 °C and then embedded in paraffin for sectioning. After mounting on glass slides, 5 μm sections were processed for immunofluorescence. The sections were first counterstained with 0.1 % Methylene Blue in PBS and mounted in Permount. The sections were then incubated in a blocking solution for 45 min at room temperature. Incubation with primary antibodies was performed in a humid chamber at room temperature for 16 h using the following dilutions in blocking solution: anti-Fpn, 1:75 and anti-HO-1, 1:100. After three washes with PBS/0.5 % BSA, the sections were incubated for 1 h at room temperature with a goat anti-rabbit-Alexa Fluor® 488-conjugated secondary antibody (Molecular Probes) diluted 1:200 in blocking solution. After washing, the slides were mounted on coverslips with anti-fading mounting reagent (Prolong Antifade kit P-7481, Molecular Probes) and examined for immunofluorescence.

Quantitative hepatic non-haem iron measurement and hepatic iron staining

Hepatic non-haem iron content was determined by acid digestion of liver samples at 65 °C for 20 h, followed by colorimetric measurement of the absorbance of the iron–ferrozine complex at 560 nm as described previously [14]. Deparaffinized liver sections were stained with Prussian Blue and counterstained with Nuclear Red using standard procedures.

Table 1 Changes in the RBC indices of 1-year-old *Sod1*$^{-/-}$ mice

Values are expressed as the means ± S.D. RBC indices were determined for ten mice of each genotype. HCT, haematocrit; MCV, mean cell volume, MCH, mean cell HB; MCHC, mean cell Hb concentration. *$P < 0.05$, significant differences between *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice

Genotype	RBC ($10^6/\mu$l)	Hb (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)
Sod1$^{+/+}$	7.2 ± 0.6	12.5 ± 1.1	39.1 ± 3.9	54.3 ± 1.6	17.5 ± 0.4	32.2 ± 1.5
Sod1$^{-/-}$	5.5 ± 0.5*	9.8 ± 0.9*	32.1 ± 3.5*	58.0 ± 2.5*	17.8 ± 0.3	30.7 ± 0.9

FACS analysis of PS (phosphatidylserine) externalization

The presence of PS on the surface of mouse RBC membranes was assessed using Annexin V–FITC staining (Calbiochem) following the manufacturer's protocol. Stained cells were analysed by flow-cytometry (FACS Calibur), and data were processed using CellQuest software (Becton Dickinson).

EP (erythrophagocytosis) assay

The EP assay was performed as described previously [15] using BMDMs (bone marrow-derived macrophages) isolated from the femurs of *Sod1*$^{-/+}$ mice and fresh RBCs obtained from 1-year-old mice of both *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ genotypes.

Haem content measurement

The haem content of formic acid-solubilized BMDMs was determined spectrophotometrically at 398 nm using haemin standards prepared in formic acid and a molar absorption coefficient of 1.5×10^5 $M^{-1} \cdot cm^{-1}$ [16].

Statistical evaluation

Where suitable, one-way ANOVA was applied to the data. The Scheffe test was used to estimate the significant difference ($P < 0.05$) between means. All calculations were performed using Statgraphics Plus 6.0 software.

RESULTS

Moderate regenerative anaemia in aged *Sod1*$^{-/-}$ mice

One-year-old *Sod1*$^{-/-}$ mice show moderate anaemia with reductions of approx. 25 % in both erythrocyte number and Hb concentration, and a reduction of approx. 14 % in the haematocrit value (Table 1). Values for the mean cell Hb concentration were unchanged, which, together with normal levels of serum iron (Figure 1A) and transferrin (Figure 1B), may suggest that the anaemia in *Sod1*$^{-/-}$ animals is not associated with iron deficiency. The raised number of peripheral reticulocytes in *Sod1*$^{-/-}$ mice (Figure 1C), as well as the increased spleen index values (Figure 1D) indicate that the anaemia in *Sod1*$^{-/-}$ mice is regenerative.

Hb and haem-scavenging systems in *Sod1*$^{-/-}$ mice

The findings obtained by Iuchi et al. [10] indicate that *Sod1*$^{-/-}$ RBCs are prone to haemolysis due to oxidative damage. Therefore we attempted first to identify hallmarks of intravascular haemolysis. We measured plasma levels of Hp and Hx, two glycoproteins involved in the clearance of Hb and haem respectively, from the bloodstream. When Hb and haem are released

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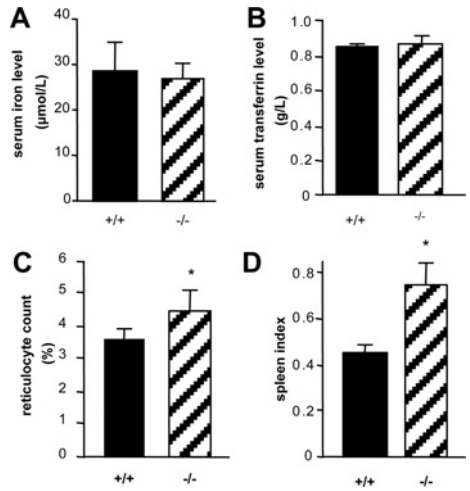

Figure 1 Evidence of regenerative, and not associated with iron deficiency, anaemia in *Sod1*$^{-/-}$ mice

Serum iron (**A**) and serum transferrin (**B**) levels in *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice. $n = 8$–10 for each genotype. (**C**) Increased number of reticulocytes in peripheral blood of *Sod1*$^{-/-}$ mice. $^{*}P < 0.05$, significant differences between values for *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice. (**D**) Increased spleen index in *Sod1*$^{-/-}$ mice. Spleen index was calculated according to the formula: spleen index $= \sqrt{100} \times$ spleen weight/body weight [45].

from ruptured erythrocytes they are instantly bound by Hp and Hx and form Hp–Hb and Hx–haem high-affinity complexes [17]. These complexes are then rapidly taken up from the circulation by CD163 [18] and CD91 [19] receptors present on tissue macrophages and hepatocytes. The rationale for measuring Hp and Hx in the plasma is that CD163 and CD91 receptors have no measurable affinity for free Hp or Hx. Thus specific recognition of Hp–Hb and Hx–haem complexes by CD163 and CD91 explains the decrease in Hp or Hx concentration in the plasma during accelerated haemolysis [19,20]. Indeed, we found that the Hp level in plasma is strongly decreased in *Sod1*$^{-/-}$ mice (Figure 2A). Surprisingly, higher plasma concentrations of Hx were detected in these animals compared with wt littermates. Considering the divergent patterns of Hp and Hx protein abundance in the plasma of *Sod1*-deficient mice, we analysed the expression the *Hp1* and *Hpx* (the mouse Hp and Hx genes respectively) genes in the liver, the major site of Hp and Hx synthesis [17]. The results shown in Figure 2(B) indicate that Hp, as well as Hx, mRNAs are markedly up-regulated. We also measured expression of CD163 and CD91 transcripts in the liver, coding for receptors of Hp–Hb and Hx–haem complexes respectively [18,19]. Levels of CD163 mRNA showed no differences between *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice, but the abundance of CD91 mRNA was significantly lower in *Sod1*-deficient mice (Figure 2C).

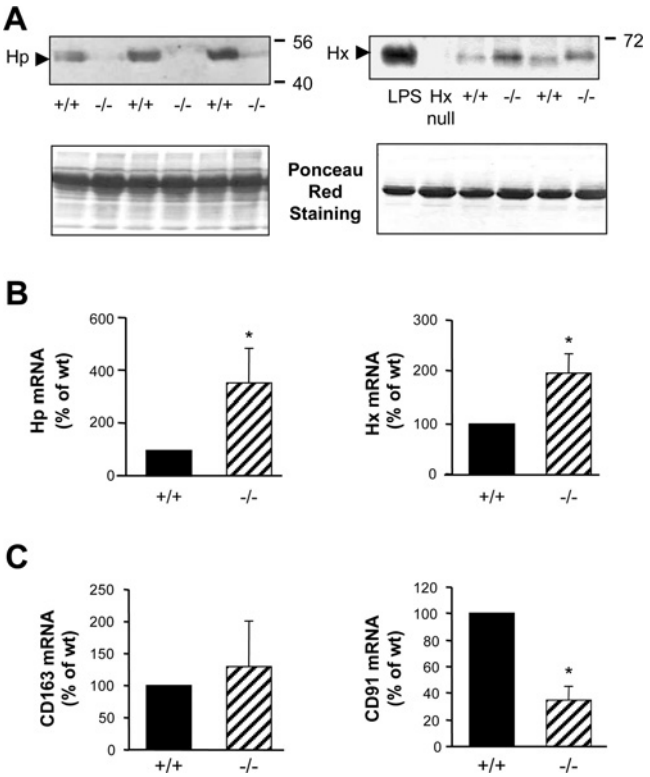

Figure 2 Changes in the expression of Hp, Hx and their receptors in *Sod1*$^{-/-}$ mice

(**A**) Plasma levels of Hp and Hx were assessed by Western blot analysis. For Hp detection, proteins from 1 μl of plasma were separated by SDS/PAGE. For detection of Hx, 2 μl of plasma was diluted in 40 μl of loading buffer and then 5 μl was loaded on to SDS/PAGE. Plasma from LPS (lipopolysaccharide)-treated *Sod1*$^{+/+}$ mice and from Hx-null mice (kindly provided by Dr E. Tolosano, University of Torino, Torino, Italy) were used as positive and negative controls respectively. Ponceau Red staining of transferred proteins is shown (bottom panels) to confirm equivalent loading. The molecular mass in kDa is indicated on the right-hand side. (**B**) Hepatic Hp and Hx mRNA levels. Transcript abundance in livers was measured by real-time RT–PCR using specific primer pairs (as shown in Supplementary Figure S1 at http://www.BiochemJ.org/bj/420/bj4200383add.htm). Each column represents the means $\pm$ S.D. of two amplification reactions, performed using a single cDNA sample reverse-transcribed from RNA prepared from four mice of each genotype. To confirm amplification specificity, the PCR products from each primer pair were subjected to melting curve analysis and subsequent agarose gel electrophoresis. The levels of Hp and Hx mRNA in *Sod1*$^{+/+}$ mice were assigned as the 100 % values ($n = 4$, $P < 0.05$). (**C**) Hepatic CD163 and CD91 mRNA levels were measured by real-time RT–PCR as described in (**B**).

Indices of extravascular haemolysis in *Sod1*-deficient mice

To investigate the susceptibility of RBCs to be removed from the circulation by EP, as a potential mechanism underlying HA, we evaluated the presence of PS externalized on the membranes of circulating RBCs. Flow-cytometric detection of externalized PS clearly showed a more than 3-fold increase in the percentage of PS-positive *Sod1*$^{-/-}$ RBCs when compared with wt RBCs (Figure 3A). In artificially aged RBCs [15], the proportion of PS translocated to the cell surface was significantly greater than the values found for *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ RBCs. We next used an EP assay to examine the correlation between the levels of externalized PS with the haem content in *Sod1*$^{-/+}$ BMDMs after phagocytosis of RBCs of the two genotypes. As expected, the haem content in BMDMs after incubation with *Sod1*$^{-/-}$ RBCs was significantly greater than after incubation with wt RBCs (Figure 3B) indicating that *Sod1*$^{-/-}$ RBCs are more readily phagocytosed than *Sod1*$^{+/+}$ RBCs.

Increase in hepatic HO-1 expression in *Sod1*$^{-/-}$ mice

To better understand the role of HO-1 during accelerated haemolysis, we analysed its expression in the liver, at both the mRNA and protein levels (Figure 4). Levels of HO-1 mRNA were increased approx. 3-fold in *Sod1*$^{-/-}$ mice compared with wt littermates (Figure 4A), and HO-1 protein levels assessed by both Western blot analysis (Figure 4B) and immunofluorescence (Figure 4C) were similarly elevated. Interestingly, HO-1 expression in liver was mostly detected in *Sod1*$^{-/-}$ KCs (Kupffer cells) in accordance with its known high expression in phagocytes. In contrast with wt mice, Hb autofluorescence was strongly present in liver sections from anaemic *Sod1*$^{-/-}$ mice (Figure 4D). Interestingly,

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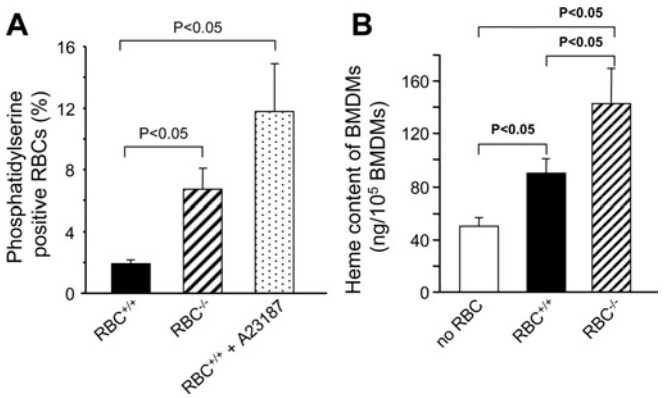

Figure 3 Indices of increased extravascular haemolysis in *Sod1*-deficient mice

(**A**) Increased externalization of PS on *Sod1*$^{-/-}$ RBCs. Exposure of PS was assessed by staining cells with Annexin V–FITC followed by flow cytometry. Artificially aged Ca^{2+} ionophore A23187-treated RBCs from *Sod1*$^{+/+}$ mice were used as control cells showing high levels of PS exposure on their cell membrane. Stained RBCs as a percentage of the total number of RBCs was calculated. Results are shown as the means ± S.D. for three wt mice and four *Sod1*$^{-/-}$ mice. Significant differences in PS exposure are indicated. (**B**) Increased haem content of BMDM after *in vitro* phagocytosis of erythrocytes. The EP assay was performed using BMDMs from *Sod1*$^{-/+}$ mice and RBCs from 1-year-old *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice. Haem content of the formic acid-solubilized BMDMs was determined spectrophotometrically at 398 nm. Values represent the means ± S.D. of four EP assays performed in duplicate on RBCs from four mice of each genotype. Significant differences in PS exposure are indicated ($P < 0.05$).

fluorescence analysis of HO-1 (green) with Hb (red) showed partial co-localization in sections of liver from *Sod1*$^{-/-}$ mice (Figure 4E).

Hepatic iron status in haemolytic *Sod1*$^{-/-}$ mice

Massive iron accumulation in tissues is one of the characteristic metabolic features of acute HAs of various aetiologies [21]. We therefore examined whether this iron overload phenotype could be observed in haemolytic *Sod1*$^{-/-}$ mice. Staining of non-haem iron in the liver showed iron accumulation in some *Sod1*$^{-/-}$ liver macrophages (Figure 5A). On the other hand, there was no evidence of global iron loading in hepatocytes. Furthermore, we observed no significant change in non-haem iron content in the liver (Figure 5B). Accordingly, a significant decrease in L-Ft protein (Figure 5C) in hepatic cytosolic extracts from *Sod1*$^{-/-}$ mice was observed. The protein level of H-Ft showed no difference between *Sod1*$^{+/+}$ and *Sod1*$^{-/-}$ mice.

Hepatic Hepc mRNA and Fpn protein levels are decreased and increased respectively in *Sod1*-deficient mice

We next studied the expression of Fpn, the only known iron exporter in mammalian cells. Western blotting of hepatic membrane extracts (Figure 6A) and immunofluorescence on histological liver sections (Figure 6B), showed that Fpn is expressed at low levels in the livers of *Sod1*$^{+/+}$ mice. In contrast, Fpn expression was markedly enhanced in the livers of *Sod1*$^{-/-}$ mice (Figures 6A and 6B). Microscopic analysis of immunofluorescence staining on liver sections (Figure 6B) identified KCs as the principal cell type of Fpn expression. Enlargement of immunofluorescent pictures also indicated the presence of RBC Hb autofluorescence in *Sod1*$^{-/-}$ KCs strongly positive for Fpn (see the enlargement in Figure 6B). At the level of mRNA, we detected a slight, but not significant, change in Fpn mRNA expression between *Sod1*$^{+/+}$ and *Sod1*$^{-/-}$ animals (Figure 6C). Considering the established role of Hepc in the degradation of Fpn molecules [22], we then analysed Hepc mRNA

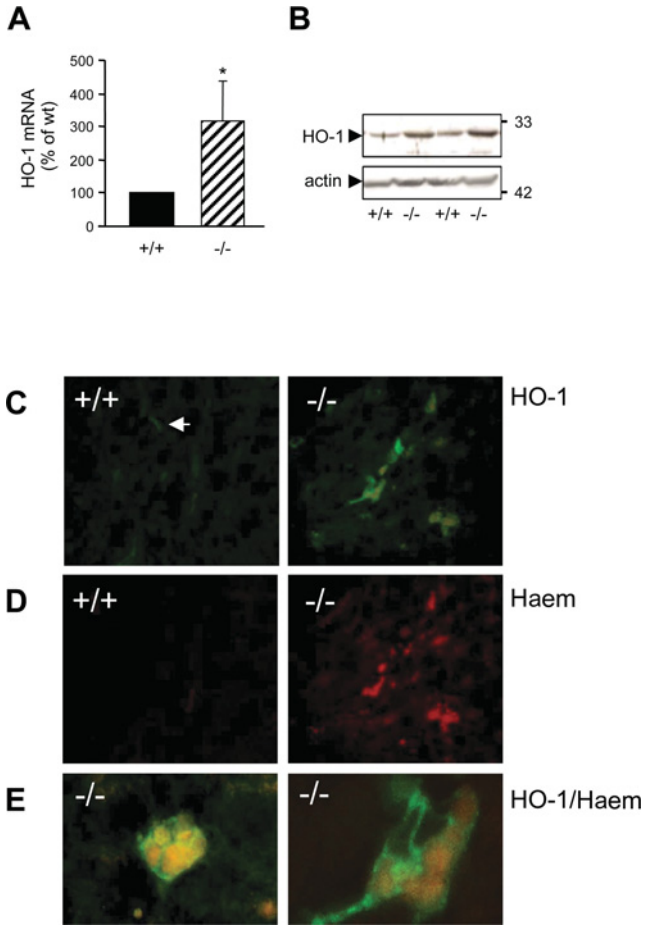

Figure 4 Increased hepatic HO-1 expression in *Sod1*$^{-/-}$ mice

(**A**) Hepatic HO-1 mRNA level. Transcript abundance in livers was measured by real-time RT–PCR using the specific primer pair shown in Supplementary Table S1 (at http://www.BiochemJ.org/bj/420/bj4200383add.htm). Reactions and presentation of results is as described in the legend to Figure 1(B). (**B**) HO-1 protein levels were assessed by Western blot analysis. Membrane proteins from liver (100 μg/lane) were separated by SDS/PAGE. An identical blot reacted with actin antibody is shown (bottom panel) to confirm equivalent loading. The molecular mass in kDa is indicated on the right-hand side. (**C**) Immunofluorescence staining of HO-1 and (**D**) autofluorescence of Hb in *Sod1*$^{-/-}$ (−/−) and *Sod1*$^{+/+}$ (+/+) livers analysed by fluorescence microscopy (original magnification 60 ×). (**E**) Enlargements of fluorescent staining in *Sod1*$^{-/-}$ liver show HO-1 expression in Hb-containing KCs.

expression in mice of both genotypes and found that, in *Sod1*$^{-/-}$ mice, the expression of Hepc mRNA was decreased by approx. 70 % (Figure 6D).

DISCUSSION

In the present study, we have investigated the consequences of oxidative stress due to the combined effects of SOD1 deficiency [4,6,10] and age-dependent alterations in redox state [4,6] on iron homoeostasis in aged mice. Considering the well-known susceptibility of RBCs to oxidative stress, and the importance of the liver in recycling haem iron, we focused the present study on the peripheral erythrocyte–liver axis. Furthermore, analysis of markers of haem-iron recycling (HO-1, Fpn) and of the macrophage phagosome maturation [Lamp1 (lysosome-associated membrane protein 1)] in the spleen of *Sod1*$^{-/-}$ mice indicates no major changes in haem-iron recycling in this tissue

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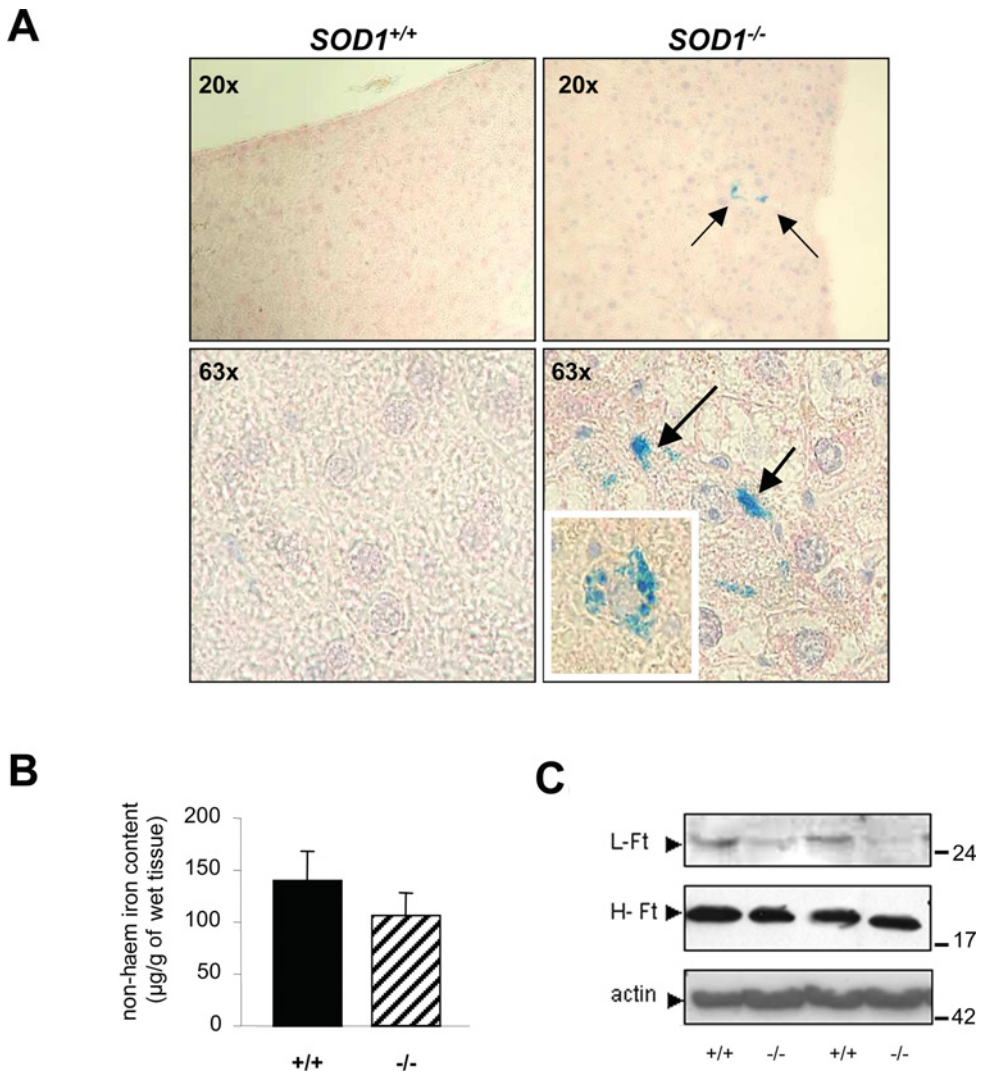

Figure 5 Hepatic iron status in *Sod1*$^{-/-}$ mice and wt littermates

(**A**) Histological examination of iron loading in liver. Non-haem iron deposits were detected by staining with Perls Prussian Blue (blue stain) exclusively in KCs of *Sod1*$^{-/-}$ mice (indicated by arrows). A high magnification of a KC presenting a clear deposit of iron, probably in Ft, is shown (inset in the bottom right-hand panel). (**B**) Non-haem hepatic iron content was measured as described in the Experimental section. Values are expressed as the means $\pm$ S.D. for liver samples obtained from ten mice of each genotype ($P < 0.05$). (**C**) H- and L-Ft levels in liver cytosolic protein extracts (50 μg/lane) were assessed by Western blot analysis. The analyses were performed in quadruplicate using extracts obtained from different *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice, and representative results are shown. The molecular mass in kDa is indicated on the right-hand side.

(Supplementary Figure S1 at http://www.BiochemJ.org/bj/420/bj4200383add.htm).

Our first observation concerning 1-year-old mice lacking Cu,Zn-SOD activity was a reduction in the values of the main RBC parameters. A similar level of anaemia has been reported in 40-week-old *Sod1*$^{-/-}$ mice [10]. It has been proposed that oxidative stress is the major factor causing SOD1-deficiency anaemia to qualify as a HA [10]. Because the mechanisms underlying HA are believed to rely on the destruction or accelerated removal of RBCs from the circulation, we looked for evidence of intra- and extra-vascular haemolysis in aged *Sod1*$^{-/-}$ mice.

Hb and haem are the most abundant iron compounds released into the plasma from ruptured RBCs and they have been implicated in toxic effects [23]. Hp and Hx, being scavengers of Hb and haem respectively, are considered plasma-protective proteins against intravascular haemolysis [17]. The increased hepatic Hp gene expression and reduced plasma Hp protein level we observed in *Sod1*$^{-/-}$ mice may reflect the induction of Hp synthesis in the liver, its release into the circulation followed by formation of the Hp–Hb complex, and its clearance by KCs via CD163 [18]. Accordingly, the plasma level of Hp is usually reduced in haemolytic states [17]. With regard to Hx, the increased levels found in the plasma of *Sod1*$^{-/-}$ mice could be due to the fact that the amount consumed by moderate haemolysis is outweighed by its enhanced production as a result of inflammation in the liver. Furthermore, the plasma Hx level starts to decrease when the Hb-binding capacity of Hp is exceeded [24]. Finally, low hepatic expression of CD91 mRNA (suggesting low protein expression) may also explain the high levels of Hx detected in the plasma of *Sod1*$^{-/-}$ mice. In support of a role for enhanced extravascular haemolysis, i.e. accelerated EP, in HA in mice lacking SOD1, we observed the presence of RBC Hb autofluorescence in liver macrophages attesting to the high EP activity of these cells, although the Hb autofluorescence signal may also derive from internalized Hp–Hb complexes. We also demonstrated marked externalization of PS on the surface

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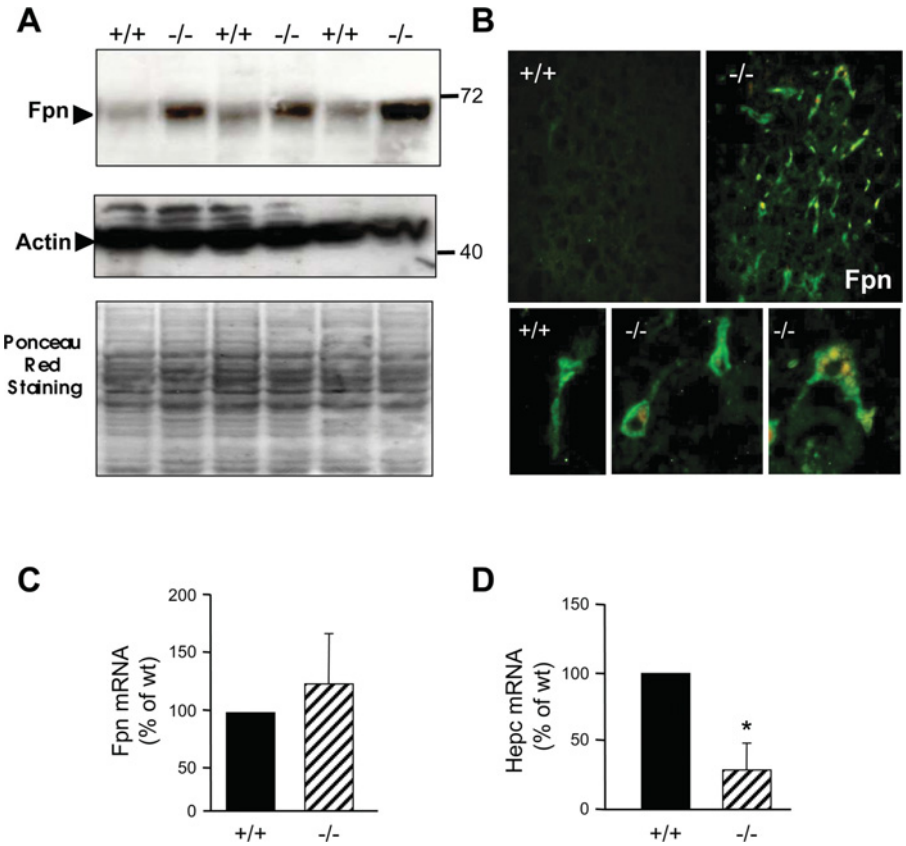

Figure 6 Correlation between enhanced Fpn and decreased Hepc expression in the livers of *Sod1*$^{-/-}$ mice

(**A**) Fpn levels in liver membrane protein extracts (100 μg/lane) as assessed by Western blot analysis. Ponceau Red staining of transferred proteins and an identical blot reacted with actin antibody are shown (bottom panels) to confirm equivalent loading. (**B**) Immunofluorescence staining of Fpn and RBCs (Hb autofluorescence) in liver sections from *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice (original magnification 63 ×). Bottom panels show enlargement of Fpn staining in KCs of wt (+/+) or *Sod1*-deficient (−/−) mice. In *Sod1*$^{-/-}$ mice, Fpn was detected in liver macrophages containing RBCs. (**C**) Expression of Fpn mRNA. Transcript abundance in livers of *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice was measured by real-time RT–PCR using the specific primer pairs shown in Supplementary Table S1 (at http://www.BiochemJ.org/bj/420/bj4200383add.htm). Reactions and presentation of the results is as described in the legend to Figure 2(B). (**D**) Expression of Hepc mRNA. Transcript abundance in livers of *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice was measured by real-time RT–PCR using the specific primer pairs shown in Supplementary Table S1). Reactions and presentation of the results is as described in the legend to Figure 2(B), except that six mice of each genotype were used. *$P < 0.05$, significant differences between Hepc mRNA levels in the livers of *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice.

of RBCs from *Sod1*$^{-/-}$ mice that probably contribute to this observation. Indeed, under physiological conditions the exposure of PS on the RBC membrane is a signal for macrophages to recognize damaged or aged RBCs [25]. A role for extravascular haemolysis in the mechanism contributing to HA is further supported *ex vivo* by the high haem content in BMDMs after incubation with RBCs from *Sod1*$^{-/-}$ mice. This observation clearly indicated the increased susceptibility of *Sod1*$^{-/-}$ RBCs to be engulfed by phagocytes.

In the present study, we characterize large changes in hepatic iron metabolism in aged *Sod1*-deficient mice displaying mild, but chronic, HA. We first analysed the expression of HO-1, an enzyme responsible for haem catabolism in macrophages [26]. We found a substantial increase in HO-1 mRNA and protein expression in the liver of *Sod1*$^{-/-}$ mice in comparison with the wt mice. In addition, HO-1 was found to be localized in KCs, which exhibited strong Hb fluorescence from ingested RBCs. In accordance with our results, increased hepatic HO-1 expression has also been reported in various haemolytic states [27,28], and in macrophages in an EP assay [15]. The role of HO-1 in systemic iron turnover has been confirmed in HO-1 deficiency [29,30]. The anaemia and hepatic iron overload observed in HO-1-deficient subjects demonstrate the importance of this enzyme in recycling haem iron. On the other hand, it has been reported that potentiated HO-1 expression does not counteract tissue iron loading in acute HA [28,31]. We found that the moderate HA occurring in *Sod1*$^{-/-}$ mice is associated with hepatic deposits of non-haem iron in KCs. Therefore the increased expression of HO-1 in *Sod1*$^{-/-}$ liver macrophages probably participates in the iron release from haem into the cytosol and its subsequent storage into Ft deposits observed in these cells. We also observed a strong up-regulation of the iron exporter Fpn in KCs that have ingested RBCs. The macrophage nature of Fpn-positive liver cells in *Sod1*$^{-/-}$ mice, has been also confirmed by immunofluorescent co-localization of F4/80 (widely used as a marker for mouse macrophages) with Fpn (results not shown). Indeed, Fpn is highly expressed in tissue macrophages, including KCs [32–34]. Hepc, a peptide secreted by the liver, binds to Fpn and causes its degradation [22]. We found that Hepc levels were reduced in *Sod1*$^{-/-}$ mice. It has been proposed that the occurrence of elevated erythropoiesis is sufficient to explain the decreased expression of Hepc by an as yet unidentified erythroid regulator negatively controlling Hepc synthesis [35]. Indeed, enhanced erythropoietic activity as a compensatory reaction in HA in *Sod1*$^{-/-}$ mice has been demonstrated by means of increased reticulocyte counts and splenomegaly (the present study, [10]). Interestingly, expansion of

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the splenic erythroid pool has been reported in erythropoietin- and phenylhydrazine-treated mice [36]. In addition, Fpn expression was shown to be enhanced after EP in macrophages [15,37]. Therefore we propose that the up-regulation of Fpn detected in KCs of *Sod1*-deficient mice is the consequence of decreased Hepc levels and enhanced phagocytosis of *Sod1*$^{-/-}$ RBCs. Although the co-ordinated down- and up-regulation of Hepc and Fpn respectively, in the liver is well-documented [38–40], its relevance for iron flux through this organ has not been clearly considered. In our mouse model of mild HA, the increased levels of hepatic Fpn protein of *Sod1*$^{-/-}$ mice may suggest iron release from the liver and in particular from KCs. In consequence, the serum iron concentration is maintained at a normal level despite increased iron demand for erythropoiesis. Consistent with this hypothesis, overexpression of Fpn in tissue culture cells was found to result in increased iron efflux and cellular iron depletion [32,37]. Of particular interest, overexpression of Fpn in J774 macrophages was associated with increase in the export of iron after EP [41]. Interestingly, no iron deposit was found in hepatocytes, and L-Ft, mostly expressed in these cells [42], tends to decrease in *Sod1*$^{-/-}$ liver. However, observations indicate that decrease of hepatic L-Ft in *Sod1*-deficient mice reflects hepatocytic secretion in response to inflammation [43] rather than iron depletion in these cells via up-regulation of Fpn. Despite clear mRNA expression of the *Fpn1* gene in hepatocytes [44], the protein expression and its subcellular localization in these cells remain poorly documented [39]. Furthermore, in mice lacking the *Hamp* (hepcidin antimicrobial peptide) gene, an up-regulation of Fpn in liver did not prevent iron overload in hepatocytes, whereas KCs were iron deficient [39,45]. Finally, in contrast with KCs, we did not detect any specific labelling of Fpn in *Sod1*$^{-/-}$ hepatocytes.

In summary, we have characterized large changes in iron and haem metabolism triggered in response to modest HA induced by oxidative stress due to SOD1 deficiency and age-related decreases in antioxidant defence. The changes act to reduce the toxicity of Hb and/or haem released from oxidatively damaged RBCs through efficient clearance of these iron compounds from the circulation. In addition, combined up-regulation of HO-1 and Fpn in KCs of *Sod1*$^{-/-}$ mice suggest that at least a part of haem iron is recycled into the circulation probably to respond to the needs of erythropoiesis.

ACKNOWLEDGEMENTS

We would like to thank Dr C. Bouton and Dr J.-C. Drapier (ICSN-CNRS,Gif-sur-Yvette, France) and Dr S. Imbeaud and Dr L. Marisa (Gif-Orsay Microarray Platform, CNRS, Gif-sur-Yvette, France) for enabling us to compare the iron-related gene expression profiles of aged *Sod1*$^{-/-}$ and *Sod1*$^{+/+}$ mice by DNA microarray technology, which was helpful for designing the experiments described in the present manuscript. We thank Olivier Thibaudeau for help in histological analysis. We thank John Gittins for critically reading the manuscript prior to submission.

FUNDING

This work was supported by the Polish Ministry of Sciences and Higher Education [grant number N30102431/0668].

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Received 30 October 2008/10 March 2009; accepted 18 March 2009
Published as BJ Immediate Publication 18 March 2009, doi:10.1042/BJ20082137

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Biochem. J. (2009) **420**, 383–390 (Printed in Great Britain) doi:10.1042/BJ20082137

online data

SUPPLEMENTARY ONLINE DATA

Haemolytic anaemia and alterations in hepatic iron metabolism in aged mice lacking Cu,Zn-superoxide dismutase

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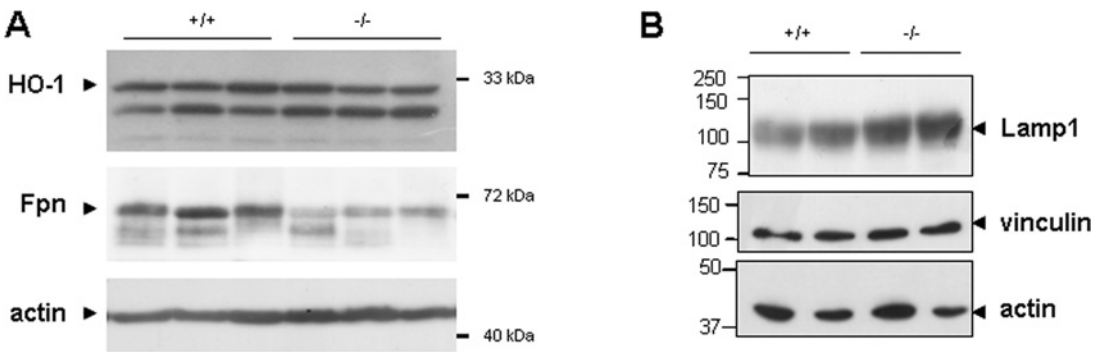

Figure S1 Expression of Fpn, HO-1 and Lamp1 in the spleen of 1-year-old knockout *Sod1* and wt mice

(**A**) HO-1 and Fpn levels in spleen membrane protein extracts as assessed by Western blotting with specific antibodies. (**B**) Lysosome-associated membrane protein 1 (Lamp1, a marker of macrophage phagosome maturation) and vinculin levels in spleen total protein extracts as assessed by Western blotting with specific antibodies (obtained from Santa Cruz Biotechnology and Invitrogen respectively).

Table 1 Oligonucleotide primers used for real-time quantitative RT–PCR analysis

CD91, haem–Hx receptor; CD163, Hb–Hp receptor; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

mRNA	Forward primer	Reverse primer	Amplified fragment (bp)	Accession number
Cd91	5′-ACGAAGCGGGTGGACGA-3′	5′-CCATCAAAAGGGCATTTATCA-3′	227	AF367720
Cd163	5′-GAAGCCTTGACAGGACAGCC-3′	5′-CATAATGAGACCCTATTGCGAAC-3′	218	AF274883
Fpn	5′-TTACATTTTCTTGCAGCAACTGTGT-3′	5′-GTTTGCAGGAGTCATTGCTGCTA-3′	80	NM_016917
Hepc	5′-CAATGTCTGCCCTGCTTTCT-3′	5′-TCTCCTGCTTCTCCTTG-3′	150	NM_032541
HO-1	5′-GTCGTGGTCAGTCAACATGG-3′	5′-TCTTGCCTGGCTCTCTTCTC-3′	253	BC010757
Hp	5′-TCTACGTGGGGAAAAACCAG-3′	5′-CATGTCATGAATGGCAAAGG-3′	25	M32599
Hx	5′-CCTGACAAAGGGAGGCAATA-3′	5′-TCTTGGCTGCATTCAGTTTG-3′	353	NM_017371

Received 30 October 2008/10 March 2009; accepted 18 March 2009
Published as BJ Immediate Publication 18 March 2009, doi:10.1042/BJ20082137

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