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Metabolism of oxidants by blood from different mouse strains

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ABSTRACT

Haemoglobins bearing reactive sulfhydryl groups have been shown to be able to interplay with glutathione in some detoxification processes. Blood from different mouse strains commonly used as experimental animal models, i.e., C57, DBA and ICR, was treated with oxidants with the aim of evaluating: (i) the involvement of protein SH groups in oxido-reductive reactions that are commonly carried out by glutathione and (ii) the impact of this phenomenon on blood-mediated metabolism of thiol reactants. All the main forms of glutathione (reduced, disulfide, and mixed disulfide with haemoglobin) were measured after oxidant treatment. Significant differences were found among the studied strains: DBA mice formed preferably mixed disulfides instead of glutathione disulfide, whereas the opposite behaviour was shown by C57 mice. Unexpectedly, the ICR strain resulted to be composed of three different subgroups (ICRa, ICRb, and ICRc), with the ICRa behaving similarly to the DBA strain, ICRc to the C57 strain, and ICRc showing an intermediate behaviour. These results are due to the different number of haemoglobin SH groups in the studied mouse strains. In particular, additional fast-reacting SH groups were found in haemoglobin from DBA, ICRa, and ICRb mice, but not in the C57 and ICRc strain. These differences were also reflected in the susceptibility of haemoglobin to dimerize and in its ability to react with S-nitrosocysteine. Because of the widely different reactivity of haemoglobin cysteinyl residues, the mouse strains examined are an interesting but complicated model in which to study the pharmacological and toxicological action of some drugs.

1. Introduction

Blood is regularly subjected to high oxygen tension and is among the first body fluids exposed to exogenous oxidative substances that are ingested, inhaled, or injected. At the same time, it possesses many different protective mechanisms by means of which a delicate balance between oxidizing and reducing species is maintained.

Defensive mechanisms include antioxidant enzymes, among which superoxide dismutase and catalase play an important role in detoxifying anion superoxide and hydrogen peroxide. Furthermore, they are provided with lipid phase antioxidants, such as α-tocopherol, and aqueous phase antioxidants such as ascorbic acid, uric acid, and glutathione [1]. Due to its high concentration, reduced glutathione (GSH) is considered the major antioxidant in blood. In addition, because

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of its nucleophilic character, GSH can trap electrophilic molecules, playing an important role in the detoxification pathways [2].

Haematic GSH concentration, under normal conditions, ranges from 1 to 1.5 mM, with similar values in blood of various mammalian (including human) and non-mammalian species [3]. Almost all GSH occurring in blood is located in erythrocytes, with plasma containing only 1–10 μM GSH. The contribution to haematic GSH concentration from white blood cells, which contain 2–4 mM GSH [4], and from platelets, with a GSH level of about 2 mM [5], is also minimal, since they represent a small volume, in comparison to red blood cells [6].

GSH is in equilibrium with its disulfide form (GSSG). The GSH/GSSG ratio can rise as a consequence of the presence of oxidants and is therefore widely studied as an index of the redox state perturbation. This balance is enzymatically controlled. The enzyme glutathione reductase reduces the disulfide bond in GSSG and therefore contributes to the cellular mechanisms for protection and repair under oxidative stress [7]. NADPH is the primary nucleotide that channels reducing equivalents to GSSG and/or other oxidants; the availability of NADPH should therefore be the ultimate determinant of cellular thiol redox status [8]. In the intact erythrocyte the supply of NADPH depends on the pentose phosphate pathway, i.e., on glucose-6-phosphate dehydrogenase activity; thus this enzyme can also be included among the antioxidant molecules [2,9].

Under pro-oxidant conditions, GSH may also reversibly bind to sulfhydryl groups of proteins (PSH) to form S-glutathionylated proteins (PSSG). PSSG can be reversed by glutaredoxin or by a non-catalysed reaction with GSH, once the restoration of an appropriate GSH/GSSG ratio is underway [10]. Glutathionylation-deglutathionylation is a dynamic process that also seems to occur under basal physiological conditions, thus contributing to the regulation of some protein functions [11]. Moreover, this process may contribute to the protection of critical PSH groups from irreversible oxidation and to exploit reducing equivalents of PSH, which may act similarly to GSH in detoxifying various oxidizing/electrophyle molecules [12]. Different pathways have been proposed for S-glutathionylation occurrence, e.g., an interaction between partially oxidized PSH (thiyl radical or sulfenic acid intermediates) and GSH or the thiol/disulfide exchange reactions between PSH and GSSG [12,13]. S-nitrosoglutathione and glutathione disulfide S-oxide are alternative mediators of PSSG formation [14,15].

Here, blood from different mouse strains, in which widely used experimental animal models of human disease have been attained by either experimental or genetic manipulation, has been treated with diamide and the Vitamin K analogue menadione (Vitamin K3) with the aim of evaluating: (i) the involvement of protein SH groups in oxido-reductive reactions that are commonly carried out by GSH and (ii) the impact of this phenomenon on blood-mediated metabolism of thiol reactants. Diamide is a widely used thiol oxidant; as a consequence of treatment with diamide, GSH usually oxidizes to GSSG and, to a minor extent, to PSSG [16]. Vitamin K3 can undergo one-electron reduction, mediated by different molecules, leading to the production of the highly reactive semiquinone, which auto-oxidizes producing redox cycling reactions. The two-electron reduced form may, instead, be conjugated to form stable sulfonated adducts (e.g., with GSH) that are unable to undergo redox cycling reactions [17]. As the mouse strains we studied are largely used in preclinical studies for testing the safety/efficacy of drugs, it is fundamental to evaluate if and to what extent blood metabolism of drugs that can interact with SH groups may be influenced by individual variations in the distribution of PSH among the different strains.

2. Methods

2.1. Materials

HPLC column Sephasil C18 (250 × 4 mm) was purchased from Pharmacia (Uppsala, Sweden). Biosil NH_2 HPLC column was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Monobromobimane (mBrB) was obtained from Calbiochem (La Jolla, CA, USA) and HPLC grade reagents from BDH (Poole, England). Diamide [azodicarboxylic acid bis(dimethylamide)], Vitamin K3 (2-methyl-1,4-naphtoquinone, menadione), and all other reagents of analytical grade were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany).

2.2. Blood collections

Blood was obtained from three different mouse strains: C57BL/6, DBA/2 and ICR (Charles River Laboratories, Calco, Lecco, Italy), homogeneous for the age (3 months). Mice were kept under controlled conditions (22–24 °C, relative humidity 40–50%) and fed ad libitum for 2–3 weeks before experiments. Blood was collected by cardiac puncture from the right ventricle of animals under light ethyl ether anaesthesia. K_3EDTA was used as an anticoagulant. All animal experimentation was conducted in conformance with the "Principles for Research Involving Animals and Human Beings" and approved by the Local Ethical Committee of the University of Siena.

2.3. Blood treatment with diamide or Vitamin K3

Aliquots of blood were treated with 1.5 mM diamide (50 mM stock solution in water) or Vitamin K3 (100 mM stock solution in ethanol) at 25 °C within 30 min from blood collection. At each indicated time, aliquots of blood were mixed with N-ethylmaleimide (NEM, 40 mM final concentration) to prevent the artifactual oxidation of sulfhydryl groups during sample manipulations [18,19] and used for GSH, GSSG, PSSG, haemoglobin (Hb) dimers, and $HbFe^{3+}$ analyses.

2.3.1. GSH, GSSG, and PSSG determinations

GSH and GSSG were measured by HPLC as previously described [19]. Briefly, a few seconds after NEM treatment, trichloroacetic acid (TCA, 5%, w/v, final concentration) was added to blood aliquots and then proteins were discarded by centrifugation (15 000 × g × 2 min). The excess of NEM was extracted from the clear supernatant with 10 vol of dichloromethane. Supernatants were then alkalinized by the addition of 1 M Tris–Cl, pH 10.0, and incubated with an equal volume of 2,4-dinitrofluorobenzene (DNFB) for 3 h at room temperature

in the dark. Samples were acidified with 1% (v/v, final concentration) HCl and detected at 355 nm after HPLC separation by means of a NH_2 column, as previously described [19].

PSSG were measured on protein pellets. NEM was removed by repeated washings with 1.5% (w/v) TCA. Successively, the protein fraction was resuspended with 0.5 mM dithiothreitol (DTT, final concentration) and 0.1 M Tris buffer, pH 8.0, and after 15 min, mBrB (2 mM, final concentration) was added. After incubation for 10 min at room temperature in the dark, samples were acidified with 5% (w/v, final concentration) TCA and centrifuged for 4 min at 15 000 × *g*. Supernatants were injected into a reversed phase HPLC Sephasil C18 column and separated under the conditions previously reported [20].

2.3.2. *Haemoglobin concentration and methaemoglobin formation*

Hb concentration was measured in samples haemolysed by 5 mM Na^+/K^+ phosphate buffer, pH 7.4 [21]. In blood samples treated with menadione, the percentage of met-Hb was calculated, at each time, by spectral deconvolution. For the calculations, both fully reduced and fully oxidized standard haemoglobins were used. Standard samples for reduced haemoglobin were obtained by the addition of 2 mg sodium dithionite to the hemolysate (before treatment with menadione) and subsequent elution with a gel-filtration PD10 column. Standard samples for met-Hb were obtained by adding to the same sample (without addition of sodium dithionite) an excess of $K_3Fe(CN)_6$. All samples were analyzed by spectrophotometer in the 500–700 wavelength range. The deconvolution analysis was performed by fitting spectra by non-linear minimization. Sigma Plot software (Jandel Scientific) was used.

2.3.3. *Electrophoresis and HPLC gel filtration*

Aliquots of blood treated with diamide and then with NEM, as above described, were centrifuged at 15 000 × *g* for 20 s to remove plasma, washed three times with 0.9% NaCl containing 2 mM NEM and then lysed by the addition of 10 vol of 5 mM Na^+/K^+ phosphate buffer, pH 6.5. After centrifugation at 15 000 × *g* for 10 min to separate membranes, supernatants were eluted through gel-filtration columns (Pharmacia, PD10)

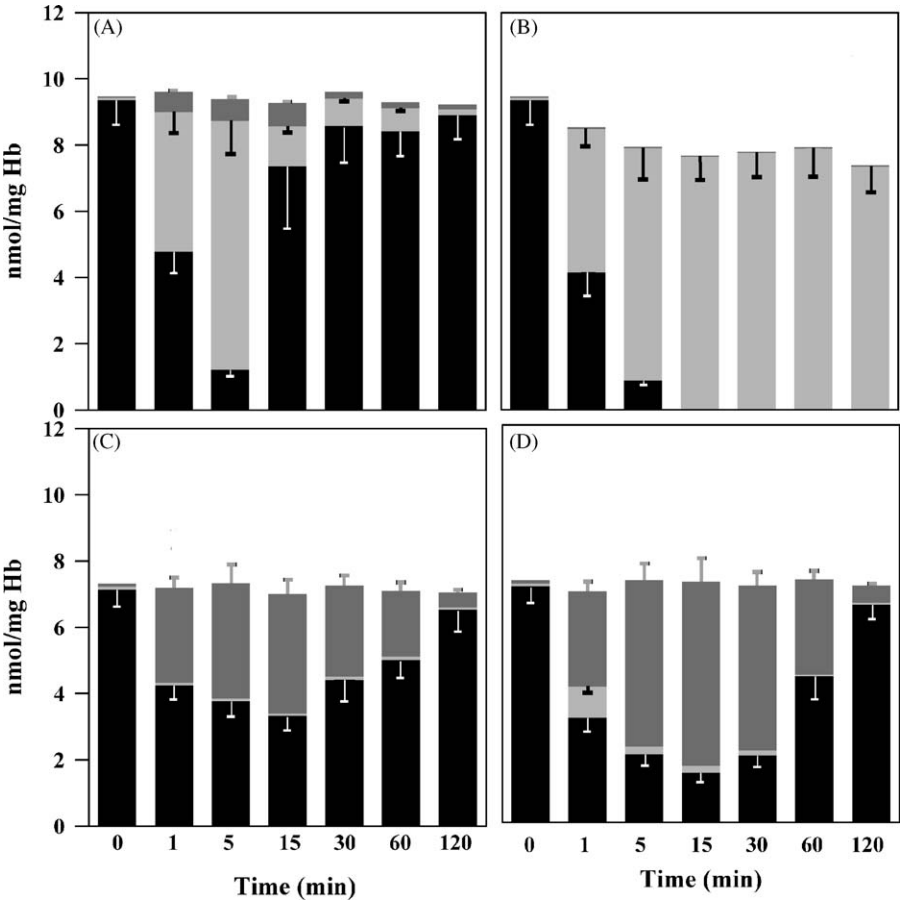

Fig. 1 – Haematic levels of GSH, GSSG, PSSG in C57 and DBA mouse strains after treatment with diamide and Vitamin K3. Time course of blood GSH (black bars), GSSG (light grey bars) and PSSG (dark grey bars) after blood treatment with 1.5 mM oxidants. Panels A and B show blood from C57 mice treated, respectively, with diamide or Vitamin K3; panels C and D show blood from DBA mice treated, respectively, with diamide or Vitamin K3. At the specified times from oxidant addition aliquots of blood were withdrawn and immediately derivatized for the analysis of GSH and GSSG by HPLC. In the ordinate, to appreciate total GSH values, levels of GSH, 2xGSSG, and PSSG are reported. White, black, and grey bars indicate S.D. values for GSH, GSSG, and PSSG levels, respectively; *n* = 5 for each strain.

equilibrated with 50 mM Na^+/K^+ phosphate buffer, pH 7.40. Aliquots of the eluate were then diluted 1:1 with sample buffer containing 5 mM NEM (final concentration) or, alternatively, 2% (v/v final concentration) β-mercaptoethanol (β-ME). Samples were analyzed by SDS (0.1%, w/v) polyacrylamide (12%) gel electrophoresis, according to the method of Laemmli [22]. The same amount of protein (20 μg) was loaded for each sample. A Mini-Protean (Bio-Rad, model 220) apparatus was used.

Alternatively, aliquots of the eluate from PD10 columns were loaded onto a 9 × 300 mm Bio-Rad Bio-Sil SEC125 column and separated isocratically by HPLC by using 0.1 M Tris–Cl buffer, pH 7.6, containing 150 mM NaCl. Tracings were followed at 280 and 540 nm wavelengths. The attribution of the molecular weight to each obtained peak was made by performing runs under the same conditions with standard proteins of known molecular weight.

2.4. Haemoglobin SH groups titration

Aliquots of untreated blood were centrifuged at 15 000 × *g* for 20 s to remove plasma, washed three times with 0.9% (w/v) NaCl and then lysed by the addition of 10 vol of 5 mM Na^+/K^+ phosphate buffer, pH 7.4. After centrifugation at 15 000 × *g* for 10 min to separate membranes, supernatants were passed through gel-filtration columns (Pharmacia, PD10) and equilibrated with 50 mM Na^+/K^+ phosphate buffer, pH 7.40. PSH were measured in the eluate by reaction with 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB, 0.25 mM final concentration) in 0.2 M Na^+/K^+ phosphate buffer, pH 6.50. The absorbance at 450 nm was recorded. Collected data were fitted to mono or multi-exponential equations by means of Sigmaplot 9.01 software (Jandel Scientific). Both amplitude and reaction rate constants were obtained from fitting results.

2.5. Enzyme assays

Aliquots of untreated blood were haemolysed by the addition of 10 vol of 5 mM Na^+/K^+ phosphate buffer, pH 7.4. Enzymatic determinations were carried out spectrophotometrically at 25 °C on hemolysates as previously described [23,24].

2.6. S-nitrosothiol analyses

S-nitrosocysteine (CySNO) was freshly prepared by mixing equimolar concentrations of 200 mM cysteine (Cys) in 0.75N HCl and 200 mM potassium nitrite in the presence of 0.1 mM diethylenetriaminepentaacetic acid. After 30 s, the solution was neutralized with 1 M Tris.

Aliquots of blood were treated with 1.5 mM CySNO (60 mM stock solution) at 25 °C. At each indicated time, aliquots of blood were mixed with NEM (40 mM, final concentration) and used for S-nitrosohaemoglobin (HbSNO) measurement. Pretreatment with NEM was applied in order to block the reaction. A few seconds after treatment with NEM, samples were centrifuged at 15 000 × *g* for 20 s to remove plasma and then washed three times with 0.9% (w/v) NaCl. Red blood cells (RBCs) were then lysed by the addition of 4 vol of 5 mM Na^+/K^+ phosphate buffer, pH 7.4. After centrifugation at 15 000 × *g* for 10 min to separate membranes, supernatants were passed through gel-filtration columns (Pharmacia, PD10) equilibrated with 50 mM Na^+/K^+ phosphate buffer, pH 7.40. HbSNO concentration was measured on eluates after decomposition of the S-NO bond with Hg^{2+} by colorimetric determination of the NO_x released as previously described [25].

All spectrophotometric determinations were carried out using a Jasco V-530 instrument.

For all HPLC measurements a Hewlett–Packard HPLC Series 1100, equipped with both diode array and fluorescence detector, was utilized.

2.7. Statistic analysis

The effects of the treatments with oxidants on blood samples with respect to basal values were statistically evaluated by applying the paired Student's *t*-test.

Differences for analyzed parameters among different mouse strains were statistically studied by the one-way ANOVA test.

The degree of correlation between GSH blood levels and the ratio SH/Hb tetramer was measured by calculating the Pearson's correlation coefficient.

3. Results

3.1. Blood treatment with oxidants: GSH, GSSG, and PSSG analysis

Blood from three mouse strains (C57, DBA, and ICR) was challenged with two different oxidants, i.e., diamide and Vitamin K3. In the C57 strain, both treatments caused a significant GSH oxidation to GSSG at all studied times

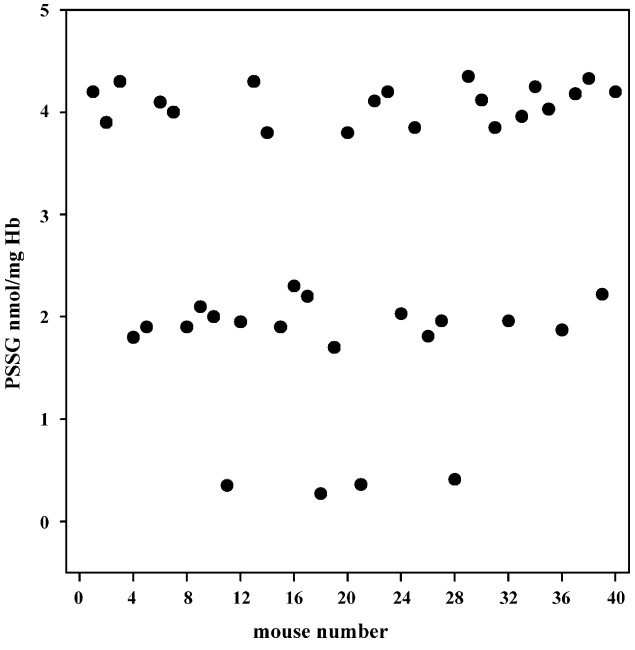

Fig. 2 – Levels of haematic PSSG in the ICR mouse strain after treatment with diamide. PSSG levels were measured after 1 min from diamide treatment (1.5 mM, final concentration). PSSG analyses were carried out by HPLC with fluorescence detection.

($p < 0.01$, with respect to untreated samples), whereas diamide alone also induced a slight, but significant PSSG formation (with respect to untreated samples, $p < 0.01$) (Fig. 1, panels A and B). In addition, Vitamin K3 provoked at all studied times a significant depletion ($p < 0.01$) of the total GSH (GSH + 2xGSSG + PSSG), likely due to GSH conjugation reactions with the quinone itself. It is known, in fact, that both Vitamin K3 and its semiquinone are able to conjugate with nucleophiles such as SH groups [26]. The formation of this conjugate was confirmed by measurement with HPLC (not shown).

Differently from the C57 strain, large quantities of PSSG ($p < 0.01$ at all studied times with respect to untreated

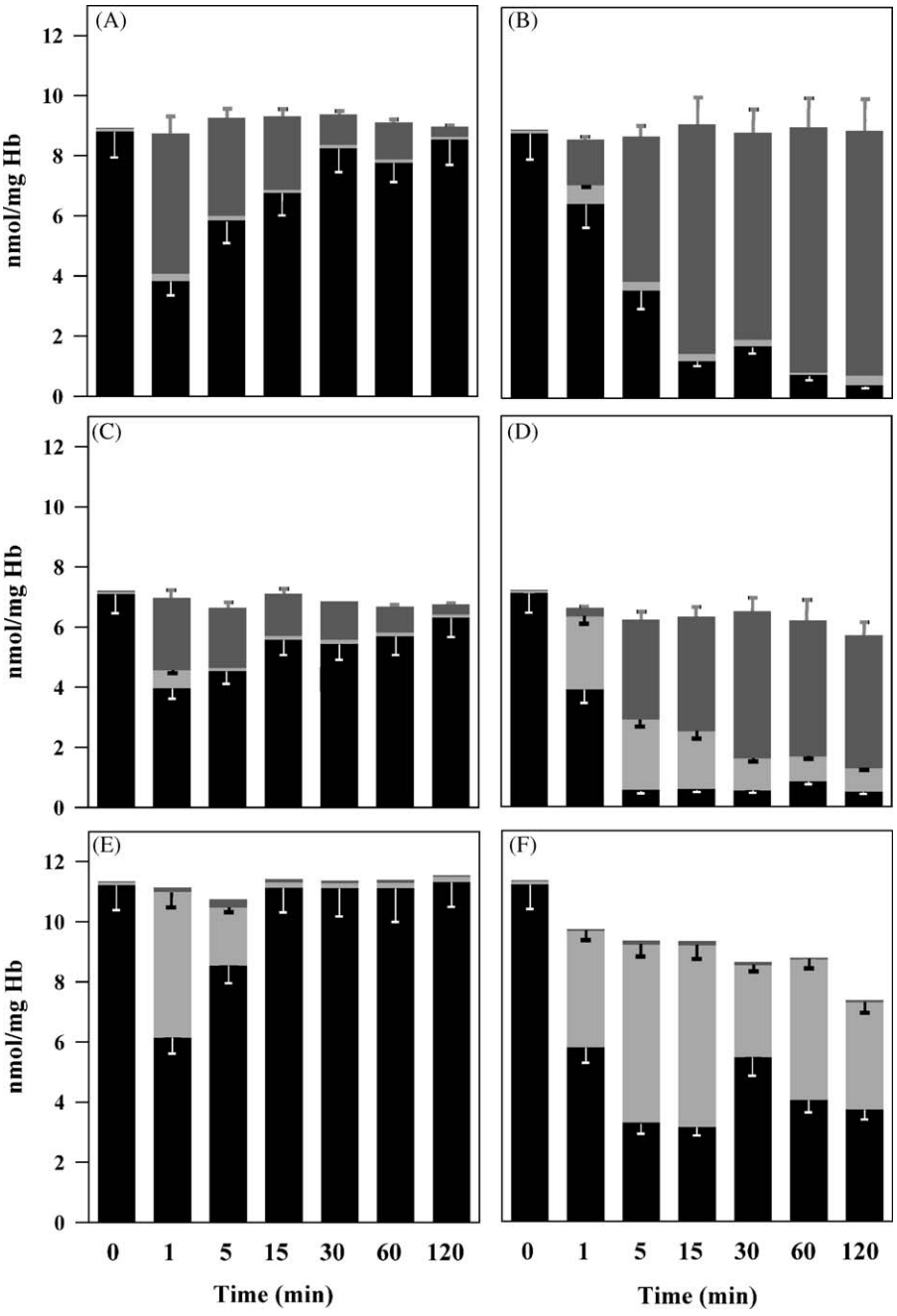

Fig. 3 – Haematic levels of GSH, GSSG, and PSSG in ICR mouse subgroups after treatment with diamide and Vitamin K3. Time course of blood GSH (black bars), GSSG (light grey bars) and PSSG (dark grey bars) after blood treatment with 1.5 mM oxidants. Panels A and B show blood from the ICRa subgroup treated, respectively, with diamide or Vitamin K3; panels C and D show blood from the ICRb subgroup treated, respectively, with diamide or Vitamin K3; panels E and F show blood from the ICRc subgroup treated, respectively, with diamide or Vitamin K3. At the specified times from oxidant addition aliquots of blood were withdrawn and immediately derivatized for the contemporaneous analysis of GSH and GSSG by HPLC. In the ordinate, to appreciate total GSH values, levels of GSH, 2xGSSG, and PSSG are reported. White, black, and grey bars indicate S.D. values for GSH, GSSG, and PSSG levels, respectively: $n = 15$ for the ICRa and ICRb subgroups; $n = 4$ for the ICRc subgroup.

samples) but not of GSSG were found, after treatment with either diamide or Vitamin K3, in blood from DBA mice (Fig. 1, panels C and D). In addition, in this strain, GSH was completely converted only to GSSG and PSSG, as evidenced by the fact that total GSH values did not significantly vary after treatment with Vitamin K3. In confirmation of this, no GS–Vitamin K3 conjugate was measured by HPLC (data not shown).

When blood of the ICR strain was tested, apparently scattered data for GSH, GSSG, and PSSG were found after treatment with the oxidants. In some mice, the formation of GSSG and PSSG was quantitatively similar to that found in the C57 strain, whereas other mice behaved similarly to the DBA strain or, alternatively, gave results not able to be correlated to previous observations. Only a significant extension of the number of the animals tested (we decided to test 40 mice) allowed us to identify three different homogeneous classes into which mice could be grouped on the basis of their responses to treatments. This particular feature is evidenced by data from Fig. 2, where the concentration of PSSG after 1 min from diamide addition is reported. Data approached three different values, approximately 4, 2, and 0.3 nmol PSSG/mg Hb. This allowed us to classify the overall population of analyzed ICR mice into three subgroups, namely, ICRa, ICRb, and ICRc, the order indicating their relative frequency: the ICRa subgroup was the most represented (about 50% of the mice analyzed belonged to this subgroup), followed by the ICRb subgroup (with about 40% of the analyzed mice) and the ICRc subgroup (about 10% of the analyzed mice). Within each subgroup, the animals showed homogeneous responses to both oxidants, as evidenced in Fig. 3.

The ICRa subgroup behaved similarly to the DBA strain after treatment with diamide and Vitamin K3 (Fig. 3, panels A and B). In fact, GSH was oxidized to PSSG but not to GSSG. A significant GSSG formation ($p < 0.01$, with respect to untreated samples) was only observed after 1 min from Vitamin K3 addition. Moreover, treatment with Vitamin K3 induced neither depletion of total GSH nor GS–Vitamin K3 conjugate (not shown).

The ICRc subgroup behaved similarly to the C57 strain after both treatments (Fig. 3, panels E and F). A significant fraction of GSH was oxidized to GSSG, whereas a negligible amount was oxidized to PSSG. In addition, GSH was partially involved in conjugation reactions with Vitamin K3 (data not shown).

An intermediate response was observed for the ICRb subgroup (Fig. 3, panels C and D). After treatment with diamide, we found a high increase in PSSG levels ($p < 0.01$ with respect to untreated samples) and also a significant production of GSSG ($p < 0.01$ with respect to untreated samples). Similarly, Vitamin K3 elicited the production of both GSSG and PSSG, the latter showing a trend to increase throughout the experiment. A minimal depletion in total GSH concentration after treatment with Vitamin K3 and a parallel increase in GS–Vitamin K3 conjugate (not shown) was measured.

3.2. *Blood treatment with Vitamin K3: Hb oxidation analysis*

Vitamin K3 metabolism is a quite complex process. It can elicit a complicated multiple oxidative pathway, being able to oxidize hemoproteins and thiols, to react with molecular oxygen, and to evoke redox cycling processes [17,27]. Therefore, in addition to the SH group, the Hb heme group can also be a target for Vitamin K3 action. In fact, Hb oxidation following pharmacological treatment with high doses of Vitamin K3 (or its aqueous soluble derivatives, e.g., Vitamin K3 sulfite) is a common life-threatening side effect [28].

The percentage of Hb oxidation was evaluated in the blood of different mouse strains treated with 1.5 mM Vitamin K3. Fig. 4, panel A shows the percentage of met-Hb found in the C57 and DBA strains. Vitamin K3 induced in both strains a rapid initial increase in met-Hb but, with time, its concentration decreased in C57 and increased in DBA blood.

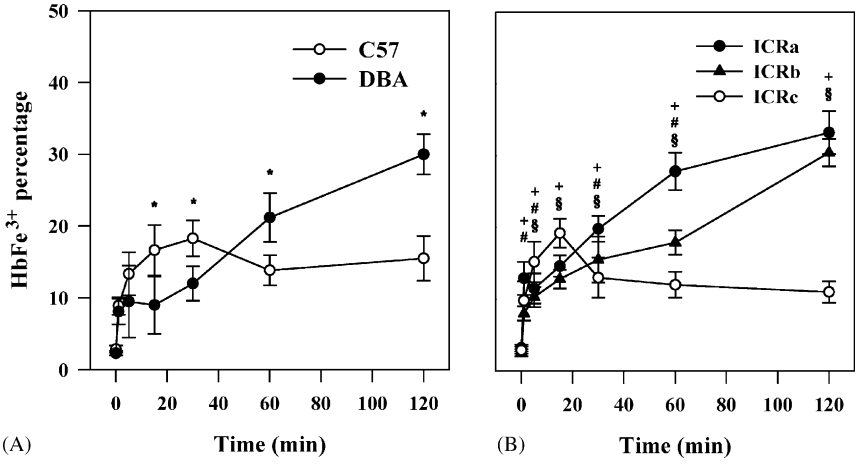

Fig. 4 – Formation of met-Hb in C57, DBA, and ICR mouse strains after blood treatment with Vitamin K3. At the specified times after treatment with Vitamin K3 (1.5 mM, final concentration), aliquots of blood were withdrawn and immediately haemolysed for spectral analysis by spectrophotometer. The percentage of met-Hb was calculated by spectral deconvolution. Data obtained for the C57 and DBA mouse strains are shown in panel A. Data obtained for the ICR strain subgroups are shown in panel B: $n = 5$ for C57 and DBA mice; $n = 15$ for the ICRa and ICRb subgroups; $n = 4$ for the ICRc subgroup. Statistic significance (ANOVA test) $p < 0.01$: *c57 vs. DBA, $^{+}$ICRa vs. ICRb, $^{\#}$ICRb vs. ICRc, and §ICRb vs. ICRc.

Results from the same experiment performed in blood from the three ICR subgroups are shown in Fig. 4, panel B. As previously observed for GSH oxidation, the C57 and ICRc strains behaved similarly, showing a rapid initial increase followed by a slow decrease. Conversely, in the ICRa strain, values of met-Hb production resembled those found in the DBA strain (i.e., a rapid initial burst phase followed by a slow continuous increase). Blood from ICRb mice displayed an intermediate behaviour between the ICRa and ICRc subgroups.

3.3. *Evaluation of Hb dimerization*

By virtue of its chemical properties, diamide is able to react with SH groups in two steps, generating disulfides (reactions (1) and (2)) [29]:

$$\mathrm{RSH} + (\mathrm{CH_3})_2\mathrm{NCON{=}NCON}(\mathrm{CH_3})_2 \rightarrow (\mathrm{CH_3})_2\mathrm{NCONSRNHCON}(\mathrm{CH_3})_2 \quad (1)$$

$$\mathrm{RSH} + (\mathrm{CH_3})_2\mathrm{NCONSRNHCON}(\mathrm{CH_3})_2 \rightarrow \mathrm{RSSR} + (\mathrm{CH_3})_2\mathrm{NCONHNHCON}(\mathrm{CH_3})_2 \quad (2)$$

The stoichiometry of the reaction indicates that one molecule of diamide oxidizes two molecules of thiol. As a consequence of blood treatment with diamide, GSSG (by oxidation of two GSH molecules), PSSG (by oxidation of one GSH molecule and one protein SH group molecule) and PSSP (by formation of disulfide bridges within or between proteins) can all be formed [16]. The prevalence of one product over the others will depend mainly on the SH reactivity and concentration as well as on the steric hindrance of the proteins involved. Therefore, we took into consideration the possibility that dimers of Hb linked by disulfide bridges (HbSSHb) could form in our samples.

RBC lysates of diamide-treated blood samples were analyzed by SDS-electrophoresis under non-reducing conditions. We searched for HbSSHb formation, testing blood samples at different time intervals within 2 h. Our results are summarized in Fig. 5: no dimers are present in control samples of all mouse strains tested (panel A), but, after treatment with diamide, a typical band around 30 kDa appeared in the DBA, ICRa, and ICRb strains (panel B). The formation of this band was not reversed with time (data not shown). In the presence of β-ME, the newly formed bands disappeared (panel B, lanes 7 and 8), thus confirming that they were due to disulfide bridge formation. Finally, we demonstrated that these disulfide bridges occurred between two Hb tetramers. This information was obtained by separating Hb samples under non-denaturing conditions (without SDS) by gel filtration with HPLC. The tracings showed that, after treatment with diamide, a new peak appeared at around 120 kDa M_W in the DBA strain (Fig. 6, panel B) as well as in the ICRa and ICRb subgroups (not shown). The new peak was not found in either the C57 strain (Fig. 6, panel A) or in the ICRc subgroup (not shown).

3.4. *Haemoglobin SH group reactivity*

Blood treatment of C57, DBA, and ICR mice with diamide and Vitamin K3 evidenced a different interplay between GSH and PSH for trapping these drugs. This could be explained by admitting the occurrence of PSH groups with different reactivity and/or concentration. Since Hb is the main source of PSH in blood, due to its high concentration, possible differences in the SH group reactivity and concentration among Hb from the different mouse strains were investigated. For this purpose, eluates obtained by gel filtration of RBC lysates were used, since Hb constitutes more than 95% of the protein content in erythrocytes, as confirmed by electrophoresis (Fig. 5).

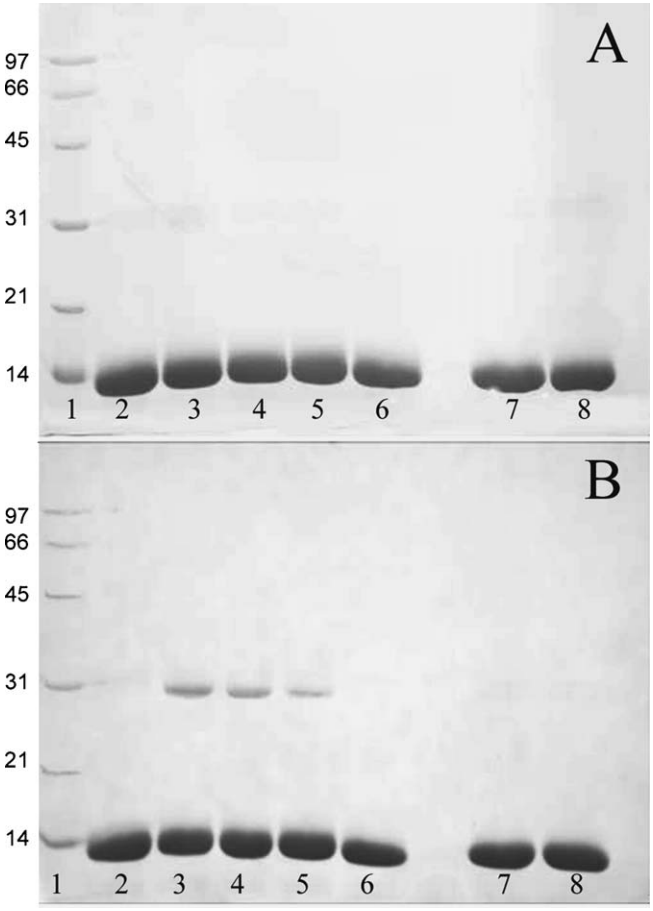

Fig. 5 – Electrophoretic pattern of RBC lysates after treatment with diamide in different mouse strains. Blood samples were treated with 1.5 mM diamide (final concentration). Aliquots of blood before treatment (panel A) or after 15 min from diamide addition (panel B) were washed in isotonic phosphate-buffered saline, pH 7.4, supplemented with glucose and NEM. Erythrocytes were lysed in ipotonic phosphate-buffered solution, pH 6.5, in the presence of NEM, deprived of membranes by centrifugation and passed through gel-filtration columns. Panels A and B: lane 1, molecular mass standards (from top to bottom: 97.4, 66.2, 45, 31, 21.5, and 14.4 kDa); lane 2, hemolysate from C57; lane 3, hemolysate from DBA; lane 4, hemolysate from ICRa; lane 5, hemolysate from ICRb; lane 6, hemolysate from ICRc; lane 7, hemolysate from DBA in the presence of β-ME; lane 8, hemolysate from ICRb in the presence of β-ME.

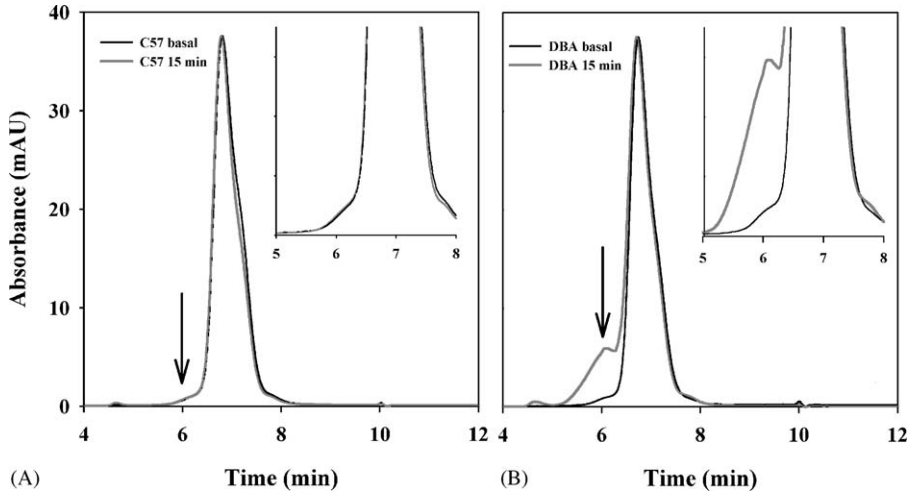

Fig. 6 – Evaluation of the inter-tetramer Hb dimerization. Blood samples were treated with 1.5 mM diamide (final concentration). Aliquots of blood before treatment or after 15 min of reaction were washed in isotonic phosphate-buffered saline, pH 7.4, supplemented with glucose and NEM. Erythrocytes were lysed in ipotonic phosphate-buffered solution, pH 6.5, in the presence of NEM, deprived of membranes by centrifugation and passed on gel-filtration columns. Eluates were, then, analyzed for the content of Hb aggregates by using a gel-filtration column for HPLC. Tracings obtained at 540 nm for the C57 mouse strain before and after 15 min of treatment are shown in Panel A. Tracings obtained at 540 nm for the DBA mouse strain before and after 15 min of treatment are shown in Panel B. The peak observed at 7 min corresponds to a molecular weight of 60 kD. The peak evidenced by the arrow at about 6 min in the DBA strain corresponds to a molecular weight of 120 kDa (an enlargement of the tracing between 5 and 8 min is shown in the inset).

The reactivity and concentration of sulfhydryl groups in Hb (Hb-SH groups) in the different mouse strains were determined by using DTNB as a titrating agent (Fig. 7). The tracings obtained differed greatly from each other. The time course of DTNB reaction with Hb-SH groups in the C57 strain and the ICRc subgroup was well fitted to a mono-exponential equation, thus indicating the presence of only one family of reactive SH groups; the calculated stoichiometry indicated that these haemoglobins contain two reactive SH groups/tetramer of Hb. The reaction between DTNB and Hb-SH in the DBA, ICRa and ICRb strains was clearly biphasic, suggesting the presence of two different families of PSH with a very different reactivity. It is deducible from our data that four SH/tetramer (two fast and two slow reacting) are present in the DBA and ICRa strains, whereas three SH/tetramer (one fast and one slow reacting) are in the ICRb subgroup. The calculated second-order rate constants and the number of SH/tetramer of Hb are summarized in Table 1.

3.5. *Enzymatic parameters*

Finally, we evaluated whether or not the variability in the Hb-SH reactivity and concentration existing among the three mouse strains could reflect on some antioxidant enzymatic activities. In particular, we focused our attention on two enzymes directly involved in the antioxidant reactions mediated by GSH, i.e., glucose 6-phosphate dehydrogenase (G6PDH) and GSSG reductase (GR). Both have a role in GSSG reduction reactions and can thus, in turn,

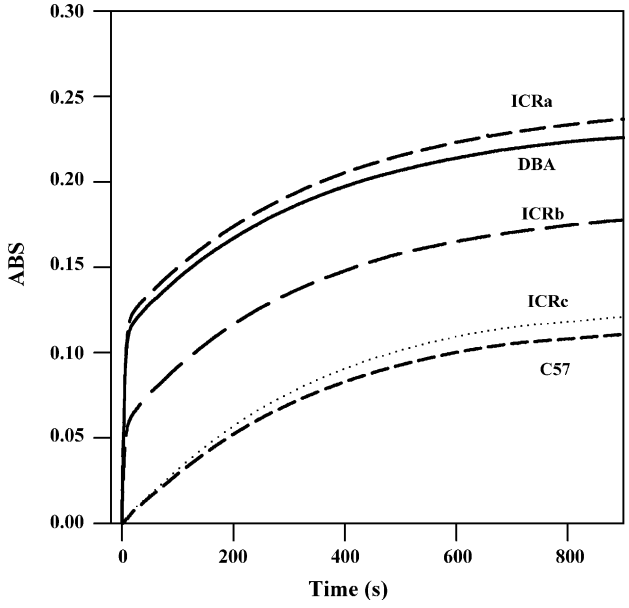

Fig. 7 – Hb-SH groups reactivity in different mouse strains. Hb of various mouse populations was treated with DTNB and the reaction was recorded by spectrophotometer at 450 nm. Haemoglobins were equimolar (15 μM final concentration, as the monomer) and were assayed at room temperature with DTNB (0.25 mM, final concentration) in 0.2 M phosphate buffer, pH 6.50. A representative experiment for each subgroup is reported.

Table 1 – Distribution and ranking according to the reactivity of Hb-SH groups in various mouse strains

	C57	DBA	ICRa	ICRb	ICRc
K_{2fast} ($M^{-1} s^{-1}$)	–	940 ± 22	1040 ± 18	980 ± 26	–
K_{2slow} ($M^{-1} s^{-1}$)	11.4 ± 0.1	11.2 ± 0.2	12.2 ± 0.2	12.6 ± 0.2	11.8 ± 0.2
SH/Hb	1.98 ± 0.03	4.06 ± 0.04	4.02 ± 0.06	2.95 ± 0.05	2.08 ± 0.04
SH_{fast}/Hb	0	1.99 ± 0.02	2.01 ± 0.04	1.03 ± 0.03	0
SH_{slow}/Hb	1.98 ± 0.03	2.07 ± 0.02	2.01 ± 0.02	1.92 ± 0.02	2.08 ± 0.04

Aliquots of untreated blood for each mouse strain/subgroup were deprived of plasma. RBC were then lysed by the addition 5 mM Na^+/K^+ phosphate buffer, pH 7.4. After membrane removing samples were passed through gel-filtration columns. PSH were measured in the eluate by reaction with DTNB at the spectrophotometer. The absorbance at 450 nm was recorded. Collected data were fitted to mono or multi-exponential equations by means of Sigmaplot 9.01 software. Both amplitude and reaction rate constants were obtained from fitting results. The number of SH are expressed per tetramer Hb. Values are shown as mean ± S.D.; $n = 4$ for each tested strain/subgroup.

regulate the thiol/disulfide status of RBCs. The activity of GR and G6PDH did not differ significantly among the mice strains considered (Table 2).

3.6. *Influence of Hb-SH reactivity on NO handling*

An example of the impact of the varying Hb-SH reactivity in different mouse strains in the handling of some thiol reactants is given by the experiments carried out with a nitric oxide (NO) donor. Large interest in the biochemistry of SH groups has been stimulated by the discovery that they can play an active role in NO metabolism and targeting through the formation of S-nitrosothiols. These molecules have been shown to undergo exchange reactions with low and high molecular weight thiols, reactions of primary importance for the final effect of NO itself [30]. In Fig. 8, the influence of the treatment of blood from different mouse strains with CySNO on the levels of RBC protein S-nitrosothiols is shown. CySNO is able to cross plasma membranes rapidly and to exchange the NO^+ group with other thiols. The kinetics of these reactions are governed by the reactivity and concentration of various thiol groups. Only slight variations in HbSNO levels were found when blood from C57 and ICRc mice was analyzed. Conversely, under the same conditions, DBA, ICRa, and ICRb blood produced statistically significant ($p < 0.01$) higher levels of HbSNO at all considered times with respect to ICRc and C57 strains.

Table 2 – Enzyme activity of G6PDH and GSSG reductase in blood of various mouse strains

	Enzyme activity (U/g Hb)	
	G_6PDH	GSSG reductase
C57	13.0 ± 3.2	4.61 ± 1.2
DBA	12.4 ± 2.5	4.47 ± 1.5
ICRa	12.3 ± 3.8	6.06 ± 1.8
ICRb	10.8 ± 3.1	6.25 ± 1.8
ICRc	10.7 ± 4.0	6.30 ± 2.2

Aliquots of untreated blood for each mouse strain/subgroup were haemolysed by the addition of 5 mM Na^+/K^+ phosphate buffer, pH 7.4. Enzymatic determinations were carried out at 25 °C spectrophotometrically on hemolysates, as described in Section 2. Values are the means of five different determinations for C57 and DBA mouse strains and ICRa and ICRb subgroups; values are the means of four different determinations for the ICRc subgroup.

4. Discussion

It has recently been recognized that PSH may also cooperate with GSH in trapping/detoxifying reactive oxygen and nitrogen species. Analogously to GSH, PSH can take part in both oxidative and electrophilic reactions [3,31–33]. Under oxidative conditions, PSH can be modified to form a cysteine sulfenic derivative, which can undergo further oxidation to sulfinic and sulfonic acid under severe oxidizing conditions. Conversely, under milder oxidative conditions, PSH can form intra-molecular or inter-molecular (PSSP) disulfide bonds

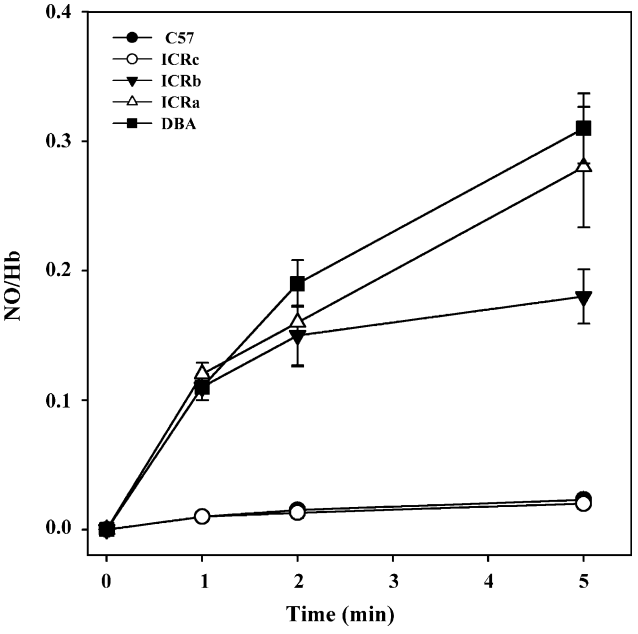

Fig. 8 – Formation of HbSNO in different mouse strains after treatment with CySNO. Blood samples were treated at room temperature with CySNO (1.5 mM, final concentration). At the indicated times aliquots of blood samples were treated with NEM for a few seconds and then plasma was discarded by centrifugation. RBCs were lysed and passed through gel-filtration columns. HbSNO concentration was measured on eluates by spectrophotometer: $n = 5$ for the C57 and DBA strains; $n = 5$ for the ICRa and ICRb subgroups; $n = 3$ for the ICRc subgroup.

and/or mixed disulfides with low-molecular weight thiols [12]. The participation of PSH in these reactions is proportional to their concentration and reactivity. The intrinsic reactivity of PSH is dependent on the pK_a value (with the anionic form much more reactive than the undissociated one) and its accessibility.

Blood is characterized by the presence of Hb at high concentration (8–10 mM), a value significantly larger than that of GSH (1–1.5 mM). Therefore, Cys residues of Hb can have a significant role in the metabolism of oxidants and electrophiles, by competing with GSH itself. It has recently been observed that Hb-SH reactivity shows significant differences between rodents and primates [3,32,34]. Specifically, human Hb has only one titrable pair of thiols per tetramer (Cys β-93), whose reactivity is slow and dependent on the quaternary conformation. Rat Hb is known to possess extra-reactive SH groups compared with human Hb, located at β-125 and α-13 positions. Cys β-125 has a low pK_a, high accessibility, and, consequently, strong reactivity, about one order of magnitude greater than that of GSH [3,32]. The occurrence of a highly reactive Cys located in position β-125 has also been observed in guinea pigs Hb [34]. On the contrary, mouse Hb does not contain Cys in position β-125, but a reactive cysteine has been found in the DBA strain in position β-13 [34].

To evaluate more in depth the possible interplay between Hb-SH and GSH in mice, we treated blood from three different mouse strains, namely C57, ICR and DBA, with two different thiol-reacting substances, i.e., diamide and Vitamin K3.

Interestingly, treatment with both oxidants elicited widely differing responses depending on the strain tested. Treatment with diamide oxidized haematic GSH, but, whereas blood from C57 mice produced mainly GSSG, that from DBA mice generated mainly PSSG (Fig. 1). Unexpected results were obtained for the ICR strain: to render the obtained data homogeneous, this strain was divided into three subgroups, i.e., ICRa, ICRb, and ICRc (Fig. 2). ICRa behaved similarly to the DBA strain, ICRc similarly to the C57 strain, whereas ICRb showed an intermediate behaviour. As a consequence of Vitamin K3 treatment DBA and ICRa produced mainly PSSG, and moreover, no GS–Vitamin K3 conjugate was found (Figs. 1 and 3); conversely, in C57 and ICRc blood, GSH was mainly oxidized to GSSG but a relevant amount of GS–Vitamin K3 conjugate was also observed. All these significant differences can be explained in the light of the experiments reported in Fig. 7. Titration of Hb-SH groups revealed that DBA and ICRa mice have four SH/tetramer, two of which are fast reacting and the other two slow-reacting SH groups. C57 and ICRc mice, on the other hand, have only two slow-reacting SH/tetramer. The ICRb subgroup, instead, was shown to possess one fast-reacting SH group/tetramer and two slow-reacting SH/tetramer (Table 1). All known mammalian haemoglobins bear two titrable SH groups in position β-93, which are characterized by a slow reactivity when titrated with DTNB. Thus, the two slow-reacting SH/tetramer we have found are likely to correspond to these residues. The fast-reacting SH, in all probability, correspond to the extra-reactive Cys located in position α-13 that has been shown to be present in some rodent strains [34]. Having large quantities of reactive cysteines, Hb from DBA and ICRa (and to a minor extent, ICRb) mice can trap diamide and Vitamin K3 and give birth to peculiar reactions. In fact, these extra highly reactive cysteines can influence Vitamin K3-derived Hb oxidation, apart from the formation of PSSG. In all probability, some percentage of Vitamin K3 is intercepted by Hb β-13 residues. This conjugate can undergo redox cycling reactions, producing reactive oxygen species, which in turn, can oxidize Hb itself. We can hypothesize that the PS–Vitamin K3 conjugate (which can form in DBA, ICRa and, to a minor extent, also in ICRb mice) is able to redox cycle more efficiently than the GS–Vitamin K3 conjugate, thus leading to the slow but continuous Hb oxidation that occurs in these blood samples after treatment with Vitamin K3 (Fig. 4).

Finally, we investigated the possibility that Hb could form inter-molecular disulfides under oxidative conditions. It is known that human Hb is not able to dimerize via S–S bridges unless denatured and with the single exception of a few mutant types of haemoglobin, e.g., Hb Porto Alegre [35]. Analogously, the inability to form dimers has also been observed in rat Hb [36]. Conversely, we found Hb dimers in some mouse strains after oxidative challenge even if in a different amount depending on the number of extra-reactive Cys occurring in the Hb molecules (see upper bands at different intensity in Fig. 5, panel B). Further investigations indicated that these dimers originate from inter-molecular bridges (Fig. 6) and are irreversible. We can infer that, due to the conformation of the dimerized tetramers, GSH and/or thioltranferases may have no access because of sterical constraints and this could be the reason why the Hb disulfide bridges cannot be reduced.

Plotting the concentration of blood GSH found in each mouse strain considered versus the number of SH groups/tetramer of haemoglobin, we found a strong inverse correlation (Fig. 9), which suggests that the lower amount of GSH present in some strains (e.g., DBA) can be related to the fact that they can exploit PSH reducing equivalents. We also verified whether this could affect some enzymatic activities (G6PDH, GSSG reductases) involved in GSH redox-regulation (Table 2), but, in this case, no difference was found among different strains. Finally, to show with an example that the presence of extra-high reactive cysteines in Hb can have a profound influence on RBCs metabolism of many molecules, we tested blood from different mouse strains for the ability to influence transnitrosation reactions. Under particular conditions, nitric oxide and NO-derived species (NO_x) can react with protein and non-protein thiols to form *S*-nitrosothiols [37]. *S*-nitroso derivatives of GSH, Cys, Hb, and albumin (and other protein or non-protein thiols) are potent, fast-acting vasodilators as well as strong inhibitors of platelet aggregation [38]. Moreover, it has been postulated that Hb-SH groups may play a role in the regulation of blood pressure by the formation of *S*-nitrosothiols [39]. After blood treatment with CySNO (Fig. 8), the intracellular levels of HbSNO rose rapidly in red blood cells from DBA, ICRa, and ICRb mice, whereas a minimal variation was found in erythrocytes from C57 and ICRc mice. This is a further demonstration of the fact that, depending on the mouse strain considered, significant differences exist for the metabolism not only of oxidants, but, virtually, of all the molecules that can react with thiol groups. Particularly puzzling is the finding that an intra-strain variability in Hb-SH groups also exists in ICR mice, although the genetic variation in protein expression in outbreed mouse strains has been previously reported [40,41]. In any case, such variability

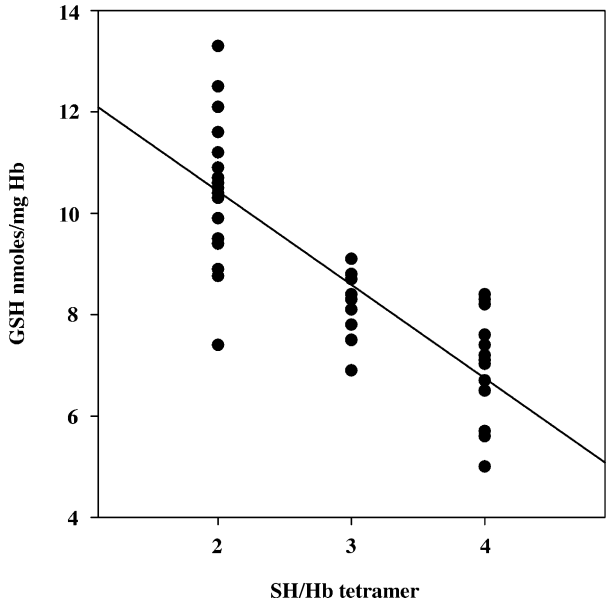

Fig. 9 – Correlation between blood GSH levels and Hb-SH group concentration. Scatter diagram is shown reporting the measured basal levels of GSH in each analyzed mouse blood sample plotted vs. the corresponding number of sulfhydryl groups per Hb molecule. Regression line, calculated by applying the Pearson correlation rank, is shown. Data from experiments indicated in Figs. 1, 3 and 7 were used.

in a fundamental and highly concentrated protein (as Hb is) must be carefully considered.

In conclusion, data obtained from our work suggest that, when mouse strains are used as an animal model for research, results can be influenced by the heterogeneous and unusual behaviour of their Hb-SH groups. Because of the widely different reactivity of these cysteinyl residues, the mouse strains we tested are an interesting but complicated model in which to study the pharmacological and toxicological action of some drugs. For example, the fact that the DBA strain and some ICR subgroups possess extremely reactive Cys residues makes their red blood cells able to metabolize some thiol-reacting substances mainly through conjugation/oxidation of Hb Cys β-13.

In any case, it should be evident to the reader that our model, being an in vitro system, presents some limitations, in that certain variables such as oxidant metabolism by tissues other than blood, the efflux of GSH and Cys mainly from liver and skeletal muscle (during starvation) and the contribution to the regeneration of reduced forms from disulfides by liver and/or other organs are not included. However, our findings suggest some hypotheses as to the possible role of haematic SH groups in the metabolism of thiolic reactants.

It is well known, in fact, that some drugs (i.e., acetaminophen, ethacrynic acid, dapsone) [42–44] have different modes of action in rodents and in man, in terms of dose-response curves and toxic limit concentrations. It is, therefore, possible that, as these compounds are able to react with SH groups, such differences may be due (at least in part) to the presence of highly reacting thiols in rodent Hb. Because the mouse is widely used as an animal model to study the haemo-toxicity of xenobiotics, we feel that our results provide a warning and can help to clarify some aspects of the metabolism of thiol reactants by mouse and human blood.

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