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Differential thiol status in blood of different mouse strains exposed to cigarette smoke

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Abstract

C57Bl/6J, DBA/2 and ICR mouse strains are known to possess different susceptibilities to developing emphysema after exposure to cigarette smoke with DBA/2 and C57Bl/6J strains being significantly more susceptible to pulmonary damage than the ICR strain. This study was aimed at analysing the occurrence of systemic oxidative stress in the blood of these different mouse strains after exposure to cigarette smoke. This study did not observe a significant decrease in glutathione in erythrocytes or in plasma cysteine, cysteinylglycine, homocysteine and glutathione in C57Bl/6J or DBA/2 mice, whereas a significant increase in the corresponding oxidized forms was observed in plasma. However, the ICR strain showed a significant increase in glutathione in erythrocytes and a significant decrease in most of the oxidized forms of cysteine, cysteinylglycine, homocysteine and glutathione in plasma after the same exposition. These experiments demonstrate that exposure to cigarette smoking induces systemic oxidative stress only in some mouse strains which are susceptible to developing emphysema.

Keywords: *Oxidative stress, thiols, emphysema*

Introduction

It is a common opinion that cigarette smoke exposure is associated with several different cardiovascular and pulmonary disorders. In confirmation of this, it is widely accepted that cigarette smoke is a risk factor for peripheral, coronary, cerebral atherosclerotic vascular diseases and for chronic obstructive pulmonary disease (COPD) or lung cancer. COPD is a condition characterized by progressive and essentially irreversible airway obstruction associated with an influx of inflammatory cells into the lungs [1]. Since the inflammation can occur in both large and small airways, heterogeneous pathological manifestations can develop in patients suffering from COPD, such as chronic bronchitis, small airway diseases and emphysema.

Although the precise pathophysiological mechanisms in which cigarette smoke (CS) can contribute to these diseases are not completely understood, particular attention has been devoted to its oxidative effects. In fact, both the gas phase and the particulate matter (tar) contain various oxidants among the more than 4700 chemical compounds found in smoke. In particular, tar is known to contain mainly stable semiquinone which can, in turn, generate H_2O_2 by the Fenton reaction, whereas in the gas phase short lived radicals, nitrogen oxides and aldehyde species such as acrolein that are able to react with thiols can be found [2–4].

The imbalance between oxidants and antioxidants in favour of the former is characteristically at the basis of the oxidative stress, which is currently under investigation since it is considered to be a potential

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contributor or, in any case, strictly related to the development/progression of many diseases. In fact, if it is not regulated properly, an excess of oxidants can damage cellular proteins, lipids or DNA, thus inhibiting signal transduction pathways and, in general, normal cellular functions [5].

Elevated levels of biomarkers of oxidative stress have been found in lungs of rats exposed to CS [6] and in the lung, exhaled breath condensate and blood of smokers [6–11]. The plasma and the epithelial lining fluid of smokers have been shown to be characterized by low levels of antioxidants such as ascorbic acid and vitamin E [12–14]. Interestingly, decreased antioxidant levels have been reported to occur even after smoking a single cigarette [15].

The occurrence of oxidative stress has been observed in patients with exacerbations of COPD [16] locally in the lung and systemically, with the hypothesis that a CS derived pro-oxidant condition has a pathogenetic role [17,18]. In any case, the administration of antioxidant therapy to smokers and/or patients affected by COPD, such as vitamin C, E or N-acetylcysteine (a precursor of glutathione), led to disappointing results [19,20]. Furthermore, it is still not known why only a small percentage of smokers acquire COPD, although >90% of patients with COPD are smokers [21]. Thus, the relationship between smoke, oxidative stress and the progression of COPD is still an open question.

In this investigation we analysed the occurrence of systemic oxidative stress in the blood of different mouse strains (C57Bl/6J, DBA/2, ICR) after 1-month of smoking by measuring the haematic balance of thiols and disulphides. These strains are known to be characterized by different tendencies in developing emphysema after exposure to cigarette smoke, DBA/2 and C57Bl/6J strains being significantly more susceptible to pulmonary damage than the ICR strain [22].

Material and methods

Blood collection

All animal experimentation was conducted in conformity with the 'Guiding Principles for Research involving Animals and Human Beings' and has been approved by the Local Ethical Committee of the University of Siena. Blood was obtained from 3 month-old C57Bl/6J, DBA/2 and ICR male mice (Charles River Laboratories, Calco, Italy). Ten animals of each strain were exposed to CS (three cigarettes/day, 5 days/week, commercial Virginia cigarettes 12 mg tar, 0.9 mg nicotine) for 1 month. Ten control mice were exposed to air under the same conditions. Blood was collected by cardiac puncture under anaesthesia with ethyl ether in plastic tubes containing K_3EDTA as an anticoagulant. Immediately after blood collection, samples were derivatized for biochemical analyses.

GSH, GSSG, GSSP determinations in RBC

An aliquot of blood samples was immediately treated with 50 mM of N-ethylmaleimide (NEM, Sigma-Aldrich, Milano, Italy, final concentration) and, after 1 min of incubation, washed three times with phosphate buffer saline solution for plasma removal. Packed red blood cells (RBC) were added 1:1 to a 20% (w/v) trichloroacetic acid (TCA) solution and proteins were separated by centrifugation. Reduced glutathione (GSH) and glutathione disulphide (GSSG) were measured by HPLC after 2,4-dinitrofluorobenzene (FDNB) derivatization, as previously described [23]. Glutathionylated proteins (GSSP) were assessed on acid precipitated proteins by HPLC and fluorescent detection with monobromobimane (mBrB, Calbiochem, La Jolla, CA), as previously described [24].

Haemoglobin determination in RBC

Haemoglobin concentration was measured in RBC hemolysed by 5 mM Na^+/K^+ phosphate buffer, pH 7.4. Samples were then analysed by a spectrophotometer (Jasco V/530) in the 500–700 wavelength range, considering the peak height at 541 nm ($\varepsilon = 13.8\ mM^{-1}\ cm^{-1}$) [25].

Thiols measurement in plasma

An aliquot of blood samples was immediately centrifuged for 15 s at 10 000 xg at 4°C for plasma separation. For plasma low molecular weight thiols (LMWSH) analyses, 50 µl of sample were deproteinized by the addition of 50 µl of 12% (w/v) TCA containing 1 mM K_3EDTA. LMWSH were analysed on the clear supernatants by HPLC after labelling with mBrB [26]. For plasma low molecular weight disulphides (LMWSS) and protein mixed disulphides (RSSP) analyses, aliquots of plasma were allowed to react for 5 min with 5 mM NEM (final concentration) and then acidified with TCA (5% w/v, final concentration) and centrifuged at 10 000 xg for 2 min. LMWSS were measured on the clear supernatants and RSSP on acidified protein pellets, as previously described [26], by using mBrB as a labelling agent. A quality control sample, constituted by 40 mg/ml bovine serum albumin dissolved in 0.1 M Na^+/K^+ phosphate buffer pH 7.4 containing 10 µM glutathione, 10 µM cysteine, 1 µM cysteinylglycine, 1 µM homocysteine, was run every 10 samples when thiols were measured.

All analyses requiring derivatization with mBrB were performed using a Spherisorb C18 column (Varian Inc, Palo Alto, CA). A Bio-Rad Biosil NH_2 (Bio-Rad, Hercules, CA) column was used for the analyses of samples derivatized with FDNB.

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All HPLC measurements were performed using an Agilent series 1100 apparatus equipped with a UV/vis and a fluorometric detector.

Statistical analysis

Data are expressed as the mean ± SD. Data obtained in control mice and after smoking were analysed using the paired *t*-student's test on commercially available software (Sigmastat, Jandel Scientific). A difference was considered statistically significant at $p = 0.05$.

Results

Influence of CS on GSH redox status in RBC

C57Bl/6J, DBA/2 and ICR mice were exposed to cigarette smoke for 1 month and then the glutathione thiol/disulphide balance was analysed in blood samples and compared with control mice. As previously reported, the analyses of GSH and its disulphide forms may represent a fine tool to investigate the oxidative status of this tissues, since they are greatly influenced by even minimal pro-oxidant conditions [27]. Data on the RBC levels of GSH and its disulphide forms (i.e. GSSG and GSSP) in the analysed mouse strains are shown in Figure1. Minimal changes were observed in the levels of GSH and its disulphides in both C57Bl/6J and DBA/2 strains after smoke exposure. However, in ICR mice the intraerythrocytic concentration of GSH increased significantly from 8.63 to 12.6 nmol/mg Hb. In this strain we also observed a significant decrease in GSSG and GSSP concentration after CS (Figure 1B and C). By calculating the ratio of the levels of the reduced and disulphides forms (i.e. GSH/(2 × GSSG) + GSSP), we observed a high thiol/disulphide ratio in all tested control strains. This condition was not influenced by CS in C57Bl/6J or DBA/2 strains, whereas it provoked a significant increase in this ratio value in ICR mice ($p < 0.01$) (Figure 2).

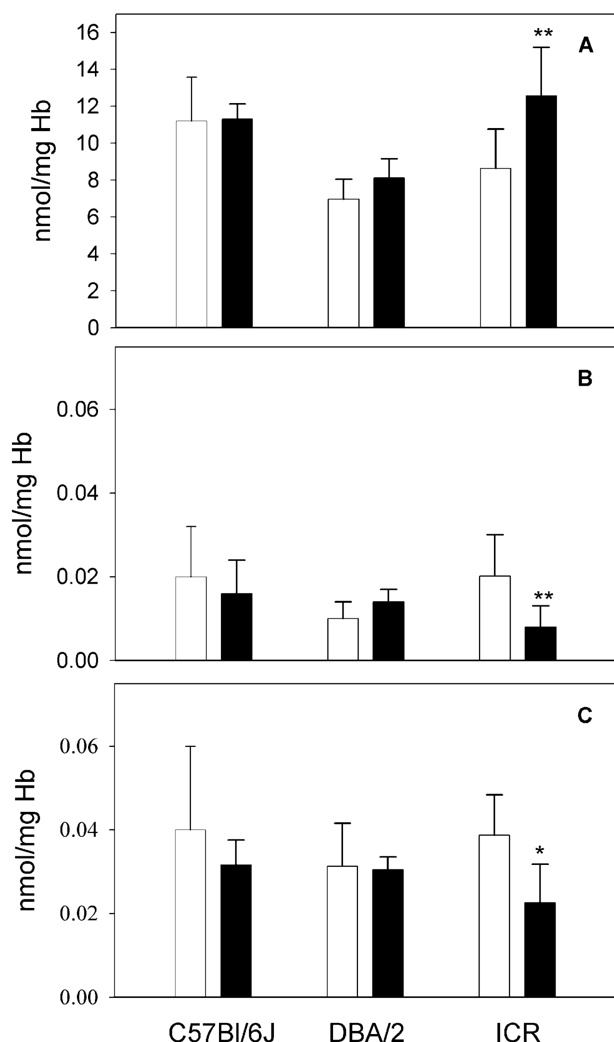

Figure 1. RBC levels of GSH (A), GSSG (B) and GSSP (C) in different mouse strains after 1 month of cigarette smoke exposure (black bars) compared to controls (white bars). RBC were immediately separated after blood collection and thiols were derivatized for HPLC analyses. Data are mean ± SD of 10 animals; $^{**}p < 0.01$; $^{*}p < 0.05$.

Thiols modulation in plasma

Differently from RBC that almost exclusively contain GSH as a low molecular weight thiol, plasma is characterized by significant amounts of other LMWSH, mainly cysteine (Cys), cysteinylglycine (CysGly) and homocysteine (Hcys) [23,25]. These thiol compounds are in balance with their oxidized forms: the low molecular weight disulphides cystine (CySS), cystinilglycine (CySSGly), homocystine (HcySS), GSSG and the corresponding mixed disulphides with proteins. The ratio between these thiols and their disulphides, as well as their relative concentration, may be influenced by pro-oxidant/antioxidant conditions. On paper, cigarette smoke exposure should have a deep impact in the plasmatic environment since it lacks reductases and the

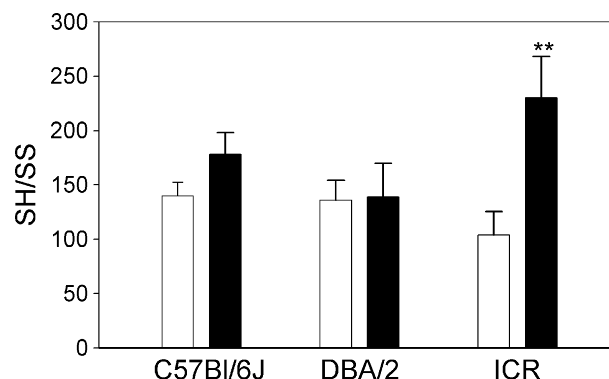

Figure 2. Thiols/disulphides ratio in RBC of different mouse strains after 1 month of cigarette smoke exposure (black bars) compared to controls (white bars). Ratios were calculated by using data from Figure 1. SS were calculated as the sum of 2 × GSSG + GSSP. Data are mean ± SD of 10 animals; $^{**}p < 0.01$.

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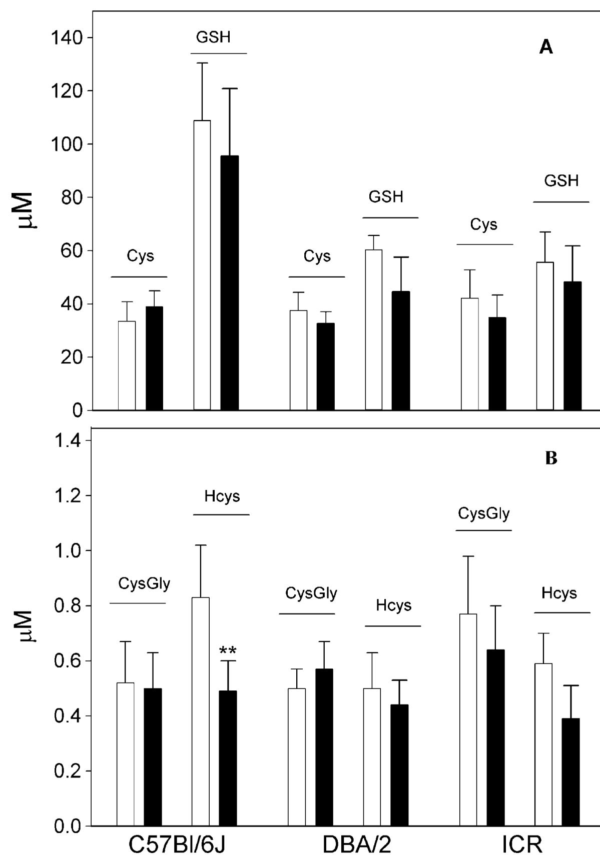

Figure 3. Plasma levels of various LMWSH in different mouse strains after 1 month of cigarette smoke exposure (dark bars) compared to controls (white bars). Plasma was immediately separated after blood collection and thiols were derivatized for HPLC analyses. Data are mean ± SD of 10 animals; ** $p < 0.01$.

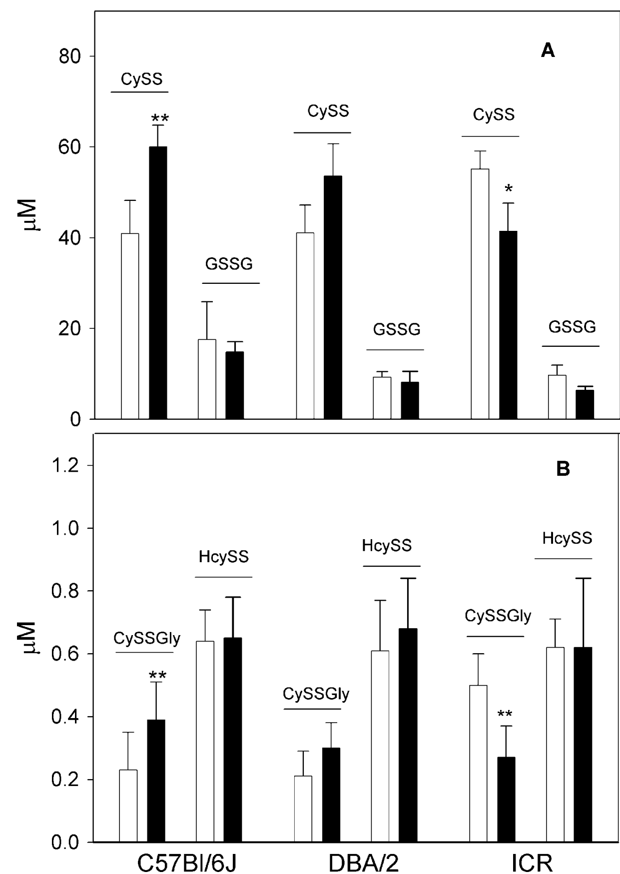

Figure 4. Plasma levels of various LMWSS in different mouse strains after 1 month of cigarette smoke exposure (dark bars) compared to controls (white bars). Plasma was immediately separated after blood collection and thiols were derivatized for HPLC analyses. Data are mean ± SD of 10 animals; ** $p < 0.01$; * $p < 0.05$.

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reduced thiols mainly derive from the intracellular compartment [28].

In our experiments, CS exposure scarcely influenced the reduced forms of these compounds in the different mouse strains (Figure 3A and B): in all tested animals plasma concentrations of Cys, CysGly Hcys and GSH did not show significant differences compared to control samples, with the only exception being Hcys in the C57Bl/6J mouse strain, which consistently decreased. Conversely, when LMWSS and RSSP were analysed in these strains, the effect of CS was more evident (Figures 4 and 5). In particular, in both C57Bl/6J and DBA/2 strains we observed an increase in various oxidized thiols, mainly in the different forms of RSSP (Figure 5). In the C57Bl/6J strain an increase in some low molecular weight disulphides, CySS and CySSGly, was also evident. In contrast, most oxidized thiols decreased in the ICR strain after CS exposure. In particular, the concentrations of all tested RSSP (Figure 5) and of CySS and CySSGly (Figure 4A and B) were significantly lower compared to controls. The effect of CS exposure on different plasma thiols and disulphides as a whole is summarized in Figure 6, in which the calculated value for total LMWSH (Figure 6A) and total disulphides (i.e. $2 \times$ LMWSS + RSSP, Figure 6B) for each studied strain is reported. Major effects were evident on disulphides, with a significant increase in these forms in C57Bl/6J and DBA/2 strains ($p < 0.01$ with respect to controls) and a significant decrease ($p < 0.01$ compared to controls) in the ICR strain. Finally, when the ratio between the total LMWSH concentration and the total disulphides concentration ($2 \times$ LMWSS + RSSP) was calculated (Figure 7), the obtained values resulted in a significant decrease from 0.799 ± 0.139 to 0.540 ± 0.024 in the C57Bl/6J strain and from 0.585 ± 0.056 to 0.288 ± 0.041 in the DBA/2 strain after 1 month of exposure to CS. This indicates that CS induces a shift towards a more oxidized thiol status in both strains and DBA/2 mice were more sensitive to the oxidative insult. Differently, the ICR strain was characterized by the prevalence of the disulphide forms in control samples (thiol-to-disulphide ratio < 0.5), whereas a significant shift towards an increase in the thiols-to-disulphide ratio was observed after CS ($p < 0.05$).

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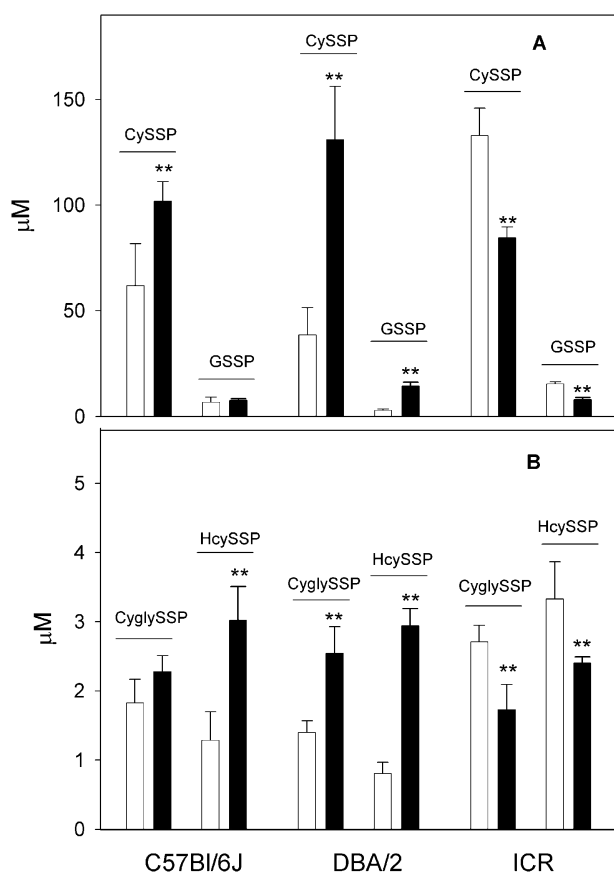

Figure 5. Plasma levels of various RSSP in different mouse strains after 1 month of cigarette smoke exposure (dark bars) compared to controls (white bars). Plasma was immediately separated after blood collection and thiols were derivatized for HPLC analyses. Data are mean ± SD of 10 animals; $^{**}p < 0.01$.

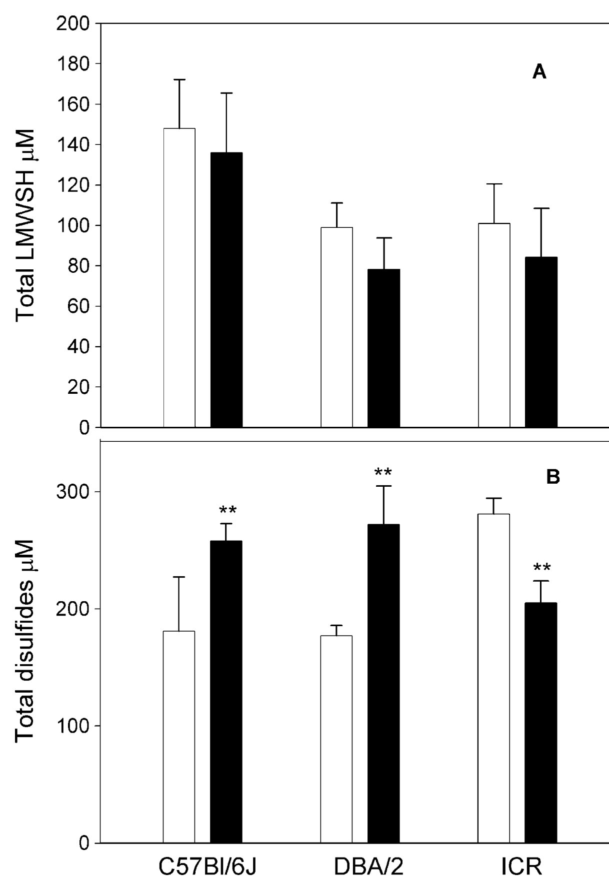

Figure 6. Plasma levels of total LMWSH (A) and total disulphides (B) in different mouse strains after 1 month of cigarette smoke exposure (black bars) compared to controls (white bars). Total disulphides were calculated as the sum of 2 × LMWSS + RSSP; data are from Figures 3–5. Data are mean ± SD of 10 animals; $^{**}p < 0.01$.

Discussion

Several different biomarkers have been studied to assess the occurrence of oxidative stress in various diseases, including COPD and related lifestyle habits such as smoking; however, some of them can be inadequate and of limited value *in vivo* because they lack sensitivity and/or specificity or their measurements are deeply influenced by sample processing or storage [5,29]. This could be one of the reasons why it is still unclear whether a relationship exists among oxidative stress, cigarette smoke and COPD. We recently demonstrated that, when correctly measured (this kind of analytical procedure is frequently plagued by several drawbacks), the redox forms of thiol compounds can be a powerful and reliable biomarker of oxidative stress status in blood [23,27]. In fact, even under slight oxidative conditions, thiols may be significantly transformed into their disulphide forms, i.e. low molecular weight disulphides and mixed disulphides with proteins. Consequently, in this paper we focused our attention on the effect of 1 month of CS exposure on hematic thiol levels in different mouse strains, selected on the basis of their known different susceptibility to developing emphysema induced by CS [22,30]. We focused our study on the hematic compartment for two main reasons. First of all, blood is able to reflect most redox alterations occurring in other tissues (generally less easily accessible for biochemical analyses) as it is continuously in contact with each of them. For example, it was reported that

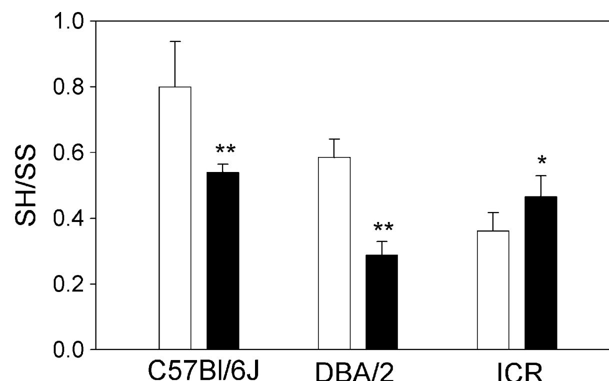

Figure 7. Thiols/disulphides ratio in plasma of different mouse strains after 1 month of cigarette smoke exposure (black bars) compared to controls (white bars). Ratios were calculated using data from Figures 3–5. SH = total LMWSH; SS were calculated as the sum of 2 × LMWSS + RSSP. Data are mean ± SD of 10 animals; $^{**}p < 0.01$.

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the antioxidant composition of respiratory tract lining fluids (RTLF) is difficult to characterize, since it varies in different levels of the respiratory tract, it is a complex biologic fluid (consisting of separate sol and gel layers) and the techniques for bronchoalveolar lavage may produce considerable variable dilution of RTLF [31]. Secondly, it is known that the RBC thiol content of these strains is typically different, which may influence the extent of oxidative perturbation after CS. In particular, we have previously observed that these strains are characterized by different concentrations of GSH and the number of reactive cysteines occurring in the β chain of haemoglobin [32]. As recently observed, the reactive β globin sulphydryl group concentration is genetically determined in mice [33]. Therefore, we expected that the occurrence of extra reactive cysteines in the haemoglobin would favour its glutathionylation under oxidative conditions and that it would play a protective role in oxidative stress mediated by CS [32,33].

In spite of the different content of erythrocytic thiols in the analysed mouse strains, CS elicited similar effects in the C57Bl/6J and DBA/2 strains, in that it did not induce an increase in glutathione disulphide and glutathionylated proteins (Figure 1B and C), which suggests that intraerytrocytic GSH and GSH replenishing enzymes successfully counteracted the CS oxidants. Cells, in fact, contain both GSSG reductase and thioredoxin, that are able to reduce the oxidized forms to GSH and, depending on the oxidative insult, these reactions can prevail over the pro-oxidant effect [34]. Interestingly, we observed an increase in the reduced glutathione in the ICR strain and a significant decrease in its oxidized forms which may have derived from a protective adaptive mechanism. It has also been reported that some components of smoke can bind to thiols, thus leading to their depletion rather than their oxidation. In particular, some aldehydes, primarily the α,β-unsatured aldehydes acrolein and crotonaldehyde, may react with GSH by generating adducts [35,36]. We can deduce that, even if these adducts can be formed, red cells are able to synthesize more GSH, thus restoring its basal levels. The ICR strain behaves differently: not only is GSH not depleted, but it is even increased, likely due to increased synthesis. It is important to note in this context that systems devoted to GSH synthesis can be up-regulated by pro-oxidant conditions [37].

Differently from the erythrocytic compartment, the extracellular redox equilibrium was more deeply modified by CS (Figures 3–5). In particular, an increase in most protein mixed disulphides in C57Bl/6 and DBA/2 strains was evidenced; in the C57Bl/6 strain we could also observe an increase in some LMWSS (CySS and CySSGly), whereas the reduced forms did not decrease significantly. This suggests that both a CS derived oxidation of thiols and their concomitant increased delivery from tissues to plasma occur in these mouse strains (Figure 6). The ICR strain showed a different thiol modulation from that of C57Bl/6J and DBA/2 mice: a slight but not significant decrease in the reduced forms and a concomitant significant decrease of most disulphides was observed. Therefore, in this strain the oxidized thiols tend to decrease after smoke, probably due to an adaptive mechanism.

In summary, CS induced a different response to the occurrence of oxidative stress in the analysed mouse strains; in particular, we found that: (i) in RBC the prevalence of reduced thiols on disulphides (high thiol-to-disulphide ratio, Figure 2) protects these cells from the occurrence of oxidative stress; in the ICR strain there is even an adaptive response which induces a further increase in GSH concentration and a decrease in disulphides levels (Figures 1 and 2); (ii) in plasma, where the thiol-to-disulphide ratio is in favour of oxidative molecules also under basal conditions, the C57Bl/6J and DBA/2 strains are characterized by significant thiol oxidation, yet in the ICR strain this ratio tends to increase as a consequence of the significant decrease in disulphides (Figures 4, 5 and 7). This is likely due to a lower presence of oxidant molecules. Briefly, the ICR strain seems to be able to counteract the CS derived oxidative insult by an increase in thiol antioxidant content in RBC and a decrease in the oxidant burden in plasma.

The different susceptibilities to developing emphysema of the analysed mouse strains [22,30] correlates well with our data regarding the ratio between oxidants and antioxidants in blood with more evident susceptibility to emphysema in the mouse strains characterized by a decrease in the thiol-to-disulphide ratio in plasma and any increase in the thiol-to-disulphide intraerythrocytic ratio (C57Bl/6J and DBA/2). Therefore, our data seems to indicate that a close relationship may exist between emphysema, a typical manifestation of COPD, and the early appearance of a perturbation of the thiol/disulphide balance. The involvement of oxidative stress and the strain dependence with effects of CS has recently been highlighted by Yao et al. [38], who found decreased levels of GSH and increased levels of lipid peroxidation products in the lungs of the C57Bl/6J susceptible strain compared with resistant strains such as ICR and 129SvJ after 3 days of CS exposure. Additional evidence for a role of oxidative stress in C57Bl/6J mice has emerged from studies that have demonstrated that transgenic C57Bl/6J mice over expressing human Cu/Zn SOD were significantly protected from CS induced emphysema [39,40]. In DBA/2 mice, which are particularly sensitive to oxidant lung injury [22], oxidative damage and apoptosis are the primary events in emphysema [30,40]. On the other hand, the resistance to oxidative stress of ICR mice has been attributed to an up-regulated Nrf2 pathway, since

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interference with the antioxidant response by deletion of Nrf2, a redox-sensitive transcription factor that up-regulates a variety of detoxification and anti-oxidant genes, caused severe smoke-induced emphysema in ICR $Nrf2^{-/-}$ mice [41].

Many different data are available regarding the occurrence of oxidative stress in patients suffering from COPD, measured locally in the lung and systemically in blood. Oxidative stress seems to mainly be due to the release of oxidants by inflammatory cells after their accumulation in the lung [17]. Additionally, it is possible that alterations in enzyme systems designed to detoxify reactive substances, such as microsomal epoxide hydrolase, glutathione transferase or cytochrome P450 iso-forms, may contribute to an increased risk for developing COPD. Finally, the up-regulation of genes coding for inflammatory cytokines could be related to a higher susceptibility to developing chronic inflammation, a key pathogenetic factor for COPD [18,42]. It has also been demonstrated that smoke can favour this condition by inducing, *per se*, oxidative stress [16]. Indeed, concentrations of isoprostanes are elevated in plasma, urine and exhaled breath condensate in patients with COPD and healthy smokers [10,43,44]). In any case, not all smokers develop COPD [21]. Similarly, in our animal models, exposure to cigarette smoking does not necessarily induce oxidative stress and characteristically only the strains in which stress occurs are more susceptible to the development of emphysema. Therefore, CS seems to be able to induce a pro-oxidant effect only in some susceptible animals. Consequently, it may be of interest to define some variables that can favour this susceptibility to developing oxidative stress after cigarette smoking. Furthermore, it is still an open question whether the altered thiol status observed in some mouse strains after CS can only be considered a good biomarker of oxidative stress or whether it may have a pathogenic role by favouring the onset of COPD as hypothesized for other pathologies [5]. In fact, it has been hypothesized that many different redox-regulated biological signalling pathways respond to changes in the thiol/disulphide redox state [45]. It has also been shown that the oxidation of the thiol/disulphide redox state is a key determinant of early events in the development of vascular diseases [46,47]. Analogously, we recently observed a modulation of this pattern occurring with ageing [26]. Therefore, the possible involvement of these redox forms in the early events of COPD may be plausible and should be investigated in the future.

The obtained data could contribute to understanding the possible link among cigarette smoking, emphysema and oxidative stress.

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Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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