Differences in lung glutathione metabolism may account for rodent susceptibility in elastase-induced emphysema development

Gisella R. Borzone,1,4 Leonel F. Liberona,1,4 Andrea P. Bustamante,1,4 Claudia G. Saez,2,4 Pablo R. Olmos,3 Andrea Vecchiola,4 Andrea Villagrán,4 Carolina Serrano,4 and Tatiana P. Reyes1,4

Departments of 1Respiratory Diseases, 2Hematology, and 3Nutrition and Diabetes, and 4Medical Research Center, School of Medicine, Pontificia Universidad Católica de Chile, Santiago, Chile

Submitted 16 April 2008; accepted in final form 9 January 2009

Differences in lung glutathione metabolism may account for rodent susceptibility in elastase-induced emphysema development. Am J Physiol Regul Integr Comp Physiol 296: R1113–R1123, 2009. First published January 14, 2009; doi:10.1152/ajpregu.90361.2008.—Syrian Golden hamsters develop more severe emphysema than Sprague-Dawley rats after intratracheal instillation of the same dose of elastase/body weight. Although species variations in antielastase defenses may largely explain these results, other variables, such as differences in lung antioxidants, cannot be overlooked since oxidative stress modulates antiprotease activity. We propose that elastase instillation might affect lung glutathione (GSH) metabolism differently in these species. Our aim was to study hamsters and rats, lung glutathione metabolism at different times, from the stage of diffuse alveolar damage to advanced emphysema. We measured total and oxidized glutathione content as well as activity and expression of enzymes related to GSH synthesis and redox cycling: γ-glutamyl-cysteine synthetase, glutathione peroxidase, and glutathione reductase. Whereas rats showed no significant changes in these measurements, hamsters showed significant derangement in GSH metabolism early after elastase instillation: 25% fall in total GSH (P < 0.05) with no increase in oxidized glutathione associated with reduced enzyme activities 24 h after elastase [60% for γ-glutamyl-cysteine synthetase (P < 0.01), 30% for glutathione peroxidase (P < 0.01), and 75% for glutathione reductase (P < 0.001)]. GSH homeostasis was restored at the end of the first week, involving transient increased expression of these enzymes. We conclude that elastase induces significant alterations in GSH metabolism of hamster lungs and no overall change in rat lungs. Although differences in disease severity may account for our findings, the hamster becomes vulnerable to functional inhibition of α1-antitrypsin by oxidants and thus, even more susceptible to injury than it would be, considering only its low α1-antitrypsin level.

diffuse alveolar damage; γ-glutamyl-cysteine synthetase; glutathione peroxidase; glutathione reductase; lung susceptibility to elastase; pulmonary emphysema

Mechanisms involved in the pathogenesis of pulmonary emphysema have been studied in animal models following elastase intratracheal (IT) instillation. A single dose of the protease in rodents induces within hours, diffuse alveolar damage with edema, hemorrhage, inflammatory cell infiltration, and rapid destruction of the extracellular matrix, resulting in airspace enlargement (25, 28, 29, 34, 39), which continues to develop over weeks and months (4, 5, 21). However, the severity of permanent lung damage after the initial injury differs among Syrian Golden hamsters and Sprague-Dawley rats. Indeed, the same dose/100 g body wt of elastase induces in hamsters a destructive lesion that resembles human panacinar emphysema, whereas in rats, the emphysema is significantly less severe (4, 5, 21), suggesting that rats have more effective protective mechanisms against elastase-induced injury.

The capacity to counteract the instilled elastase in animal models is mostly dependent upon the activity of α1-antitrypsin (α1-AT), the main lung antiprotease (28). Species differences in antielastase activity by α1-AT might explain the development of a more severe emphysema in the hamster, since this animal has a lower serum and lung α1-AT concentration compared with the rat, as we (5) and others (19) have shown. However, species variations in the amount of antiprotease defenses might not be the only mechanism involved. Other factors, namely those affecting functional activity of antiproteases, cannot be overlooked. In this context, the role of antioxidants has not been studied. Oxidative stress is known to diminish α1-AT affinity for elastase, by oxidation of a methionine residue at its active site, leaving more free elastase to interact with lung matrix components (9, 10, 11, 20, 26), thus worsening the elastase-induced imbalance between proteases and antiproteases (9, 10, 11, 20, 26).

The large initial inflammatory reaction and the persistent mild inflammatory cell infiltrate in the lungs during late stages of elastase-induced emphysema (4) represent an important source of reactive oxygen species that could alter oxidant/antioxidant balance and contribute to redox regulation of antiprotease function, depending on the antioxidant reserve of each species.

Recent evidence for the modulation of elastase-induced lung damage by antioxidants has been provided by Rubio et al. (33) who reported the attenuation of elastase-induced emphysema in rats after oral administration of N-acetylcysteine, a well-known glutathione (GSH) regenerator. GSH and its related enzymes form part of the main antioxidant system for the maintenance of intracellular redox balance (32). Significant changes in GSH metabolism that include upregulation of several genes encoding GSH-related enzymes have been reported in the lungs of humans and animals exposed to cigarette smoke, a well-known oxidant source (14, 17, 27, 31, 43). In contrast, GSH metabolism in the lungs of elastase-treated animals (i.e., lacking the participation of exogenous oxidants) has not been studied.

We hypothesized that there are changes in GSH metabolism in the lungs of hamsters and rats after IT instillation of elastase.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
and that these changes are species specific and affect more the hamster than the rat lung, thus enhancing the species differences in elastase inhibitory capacity due to differences in α1-AT concentration.

The aim of our work was to study several aspects of glutathione metabolism in the lungs of rats and hamsters prior to and after elastase instillation. In basal conditions and at different time points after elastase instillation, we measured lung total and oxidized glutathione (GSSG) content and the activity and expression of the rate-limiting enzyme in glutathione synthesis: γ-glutamylcysteine synthetase (γ-GCS) also named γ-glutamylcysteine ligase (γ-GCL), as well as the activity and expression of the enzymes involved in GSH redox cycle: glutathione peroxidase (GPx) and glutathione reductase (GRd).

Notwithstanding the control of factors like cigarette smoking, a large variability exists in chronic obstructive pulmonary disease development among human subjects with α1-AT genetic deficiency, reflecting the fact that in many cases, there are other mechanisms besides low levels of the antiprotease that contribute to emphysema development, although they remain poorly understood. The identification of an oxidant-antioxidant imbalance contributing to worsen the effects on the lung of a low level of α1-AT in a model that lacks the oxidant burden of cigarette smoke, could allow the design of studies using antioxidants to protect functional activity of α1-AT in susceptible individuals with low levels of the antiprotease. In addition, since several types of evidence suggest that genetic modifiers of α1-AT deficiency do exist, it could be possible to study genes involved in GSH metabolism as potential genetic factors relevant to variable disease expression in individuals with α1-AT deficiency as well as in chronic obstructive pulmonary disease unrelated to this deficiency.

METHODS

Animal Handling

This study was performed in adult male Sprague-Dawley rats and Syrian Golden hamsters (pretreatment body weight: 250 ± 10 g and 100 ± 10 g, respectively) in accordance with guidelines from the Animal Care and Use Committee at our institution. The protocol was approved by the School of Medicine Ethics Committee.

Under anesthesia with chloral hydrate (45 mg/100 g body wt ip), a small cervical skin incision was performed to separate the strap muscles and expose the trachea, which was punctured with a 25-gauge needle for the administration of the pancreatic elastase solution (0.55 U/100 g body wt, in 0.5 or 0.3 ml of 0.15 M sterile NaCl, respectively; Sigma, St. Louis, MO). After recovery from anesthesia, animals were fed ad libitum and maintained in the animal care facility with a 12:12-h light-dark cycle.

For both species, three groups of animals (n = 5 each) without receiving elastase, maintained for the same period of time under similar conditions, served as controls: 1) controls without intervention, 2) controls undergoing cervical skin incision, and 3) controls undergoing cervical skin incision and IT instillation of saline solution.

Animals were killed with an overdose of intraperitoneal chloral hydrate, at 4, 12, and 24 h and 4, 7, 15, 30, and 60 days after elastase (n = 8 per group after 20% lethality rate postelastase). In rapid succession, the animal abdomen was opened, and the vena cava was sectioned to allow for exsanguination, prior to freeze-clamping of the right lung, using liquid nitrogen and storage at −80°C.

Evaluation of Disease Severity

In another group of animals, the left lung served to obtain the dry weight-to-wet weight ratio, whereas the right lung underwent bronchoalveolar lavage to measure total protein content, hemoglobin content, total GSH level, and immunoreactive α1-AT by Western blot analysis (n = 8 at 4 h and 24 h and 4 days and 7 days).

Total and Oxidized Glutathione Content

Lung tissue samples were homogenized in 5% sulfosalicylic acid to produce 10% homogenates. After centrifugation, total glutathione content was immediately measured using the DTNB-GSSG reductase recycling assay, modified by Griffith (15). Results were expressed as nanomoles of GSH equivalents per gram of wet weight either as absolute values or as percentage of control. For GSSG, derivatization with vinylpyridine was used, and the assay run in the same way as for total glutathione. Results were expressed as nanomoles of GSH equivalents per gram of wet weight and as the GSSG-to-total GSH ratio. Total GSH content in bronchoalveolar lavage fluid (BALF) was expressed as nanomoles per milliliters of recovered fluid.

Enzyme Activities

γ-GCS equals γ-GCL. Lung tissue samples were rapidly homogenized in 1:10 buffer Tris-HCl, pH 8.0. After centrifugation, the activity of γ-GCS was measured in the supernatant, using the coupled assay with pyruvate kinase and lactate dehydrogenase, according to the method described by Seelig and Meister (35). The rate of reduction in absorbance at 340 nm and 37°C was followed. Enzyme-specific activity was defined as micromoles of NADH oxidized per minute per milligram of protein, which is equal to 1 IU. Results were expressed as international unit per milligram protein, either in absolute values or as percentage of control values.

Enzymes of the glutathione redox cycle. Lung tissue samples were homogenized (1:10) in 10 mM potassium phosphate buffer pH 7.4 supplemented with 30 mM KCl. After centrifugation, the supernatant was stored at −80°C for prompt determination of enzyme activity.

GPx activity was assayed according to Gunzler et al. (16) at 37°C, using tert-butyl hydroperoxide as substrate, avoiding problems contributed by catalase in lung homogenates. Results were expressed as milliunits per milligram protein, either as absolute values or as percentage of controls.

GRd activity was assayed by the method of Smith et al. (37), based on the reduction of DTNB by GSH at 24°C. Results were expressed as milliunits per milligram protein, either as absolute values or as percentage of control values.

Total protein concentration in lung homogenates and in BALF was determined by the method of Bradford (6). Hemoglobin content in BALF was quantified using light absorbance at 414 nm and the molar extinction coefficient of hemoglobin.

mRNA Expression of GSH-Related Enzymes During the Recovery Phase of Glutathione Homeostasis

RNA extraction and reverse transcription. Total RNA was isolated using Trizol B (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Reverse transcription was performed in 20 μl total volume using 2 μg total RNA, 10 U Moloney marine leukemia virus reverse transcriptase, 10 nm each of dNTPs, 10 U RNasin and oligo(dT)s as primers. All reagents were from Promega (Southampton, UK).

Semiquantitative real-time RT-PCR. Expression levels for the mRNAs of the target genes (γGCS, GPx, and GRd) normalized to an internal control, 18S ribosomal RNA (18S), were monitored over the first 7 days after elastase instillation. PCR was performed using specific primers for γGCS, GPx, GRd, and 18S (Table 1) in a reaction mix containing HotStart Taq DNA polymerase in the QuantiTect SYBR Green PCR Master Mix (Roche, Indianapolis, IN). Levels of mRNAs were determined using a LightCycler (Roche Diagnostics, Germany).
GLUTATHIONE AND ELASTASE-INDUCED LUNG INJURY

RESULTS

Species Differences in Basal Lung Glutathione Metabolism (Control Groups)

When comparing the three control groups for each of the species, values for GSH and GSSG content and for the GSH-related enzyme activities were not significantly different. Thus data from control animals were analyzed as a single group.

Total and oxidized glutathione content. Table 2 summarizes the values obtained for total and oxidized glutathione content in the lungs of control hamsters and rats. The hamster lung showed a significantly higher total glutathione content than the rat lung ($P = 0.03$). The higher total glutathione content found in the hamster lung is mainly due to a higher GSH content, since neither GSSG content nor the GSSG-to-total GSH ratio were significantly different between hamsters and rats.

Enzyme activities. Table 2 also shows that hamsters and rats have marked differences in their basal GSH-related enzyme activities. γ-GCS, the rate-limiting enzyme in glutathione synthesis, is less than one-half as active in the hamster lung as it is in the rat lung ($P < 0.001$). In relation to the glutathione redox cycle enzymes, while the activity of Gpx is significantly lower in hamster than in rat lung ($P < 0.001$), Grd activity is higher in hamster than in rat lung ($P < 0.001$). Figure 1 depicts the most important regulators of glutathione availability in hamster and rat lung cells. The high Grd activity enables hamster lung cells to maintain their reducing environment efficiently, rendering them less dependent on regulation via their GSSG efflux system. Thus, the hamster lung cells might be less dependent on GSH synthesis to sustain intracellular GSH levels. In comparison, rat lung cells appear to regulate their intracellular reducing environment by increased GSH synthesis to compensate for a less-effective GSSG reduction by Grd.

Species Differences in Lung GSH Metabolism After IT Elastase Instillation

Time course of changes in total and oxidized glutathione content. Figure 2 illustrates the changes in tissue total, reduced, and oxidized glutathione content after elastase instillation into the lungs of hamsters and rats.

In hamsters, 4 h after elastase, total glutathione levels were still within control values. At 12 and 24 h there was a 25% drop in total glutathione content ($P < 0.05$, Fig. 2A), followed by a slow and progressive recovery to control values by the second week. With a similar time course, a 50% fall in GSSG content occurred 12 and 24 h after elastase ($P < 0.01$, Fig. 2E), with values within the control range by the end of the second week. In contrast, in rats no significant variation

Table 1. Primers and product sizes for RT-PCR

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sense</th>
<th>Primer Antisense</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-GCS</td>
<td>5'-tggagacccgtatggaag-3'</td>
<td>5'-tctgtggctttggttg-3'</td>
<td>218 bp</td>
</tr>
<tr>
<td>Gpx</td>
<td>5'-agttccacctgtgtgccc-3'</td>
<td>5'-gaatccgctggcaggg-3'</td>
<td>205 bp</td>
</tr>
<tr>
<td>GRd</td>
<td>5'-caagtctaaagggcttctag-3'</td>
<td>5'-cattgctttcctctgttg-3'</td>
<td>292 bp</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>5'-acgaggaagccaccagaga-3'</td>
<td>5'-acacccacccacggatag-3'</td>
<td>158 bp</td>
</tr>
</tbody>
</table>

Table 2. Species differences in lung basal glutathione metabolism

<table>
<thead>
<tr>
<th></th>
<th>Hamsters</th>
<th>Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total GSH, nmol/g wet tissue</td>
<td>1947±60</td>
<td>1436±43*</td>
</tr>
<tr>
<td>GSSG, nmol/g wet tissue</td>
<td>87±6</td>
<td>79±8</td>
</tr>
<tr>
<td>GSSG/Total GSH ratio, %</td>
<td>4.5±0.3</td>
<td>5.5±0.5</td>
</tr>
<tr>
<td>γ-GCS, IU × mg prot⁻¹</td>
<td>4.0±0.1</td>
<td>8.8±0.9†</td>
</tr>
<tr>
<td>Gpx, U × mg prot⁻¹</td>
<td>386±9</td>
<td>598±42†</td>
</tr>
<tr>
<td>GRd, μU × mg prot⁻¹</td>
<td>90.9±2.4</td>
<td>65.9±2.9†</td>
</tr>
</tbody>
</table>

GSH, Reduced glutathione; GSSG, oxidized glutathione; γ-GCS, glutamylcysteine synthetase; prot, protein; Gpx, glutathione peroxidase; GRd, glutathione reductase. *$P < 0.05$; †$P < 0.001$.
Fig. 1. Schematic overview of glutathione metabolism in hamster and rat lungs at baseline. Left: by comparison with the rat, the steady-state concentration of reduced glutathione (GSH) in the hamster lung seems to depend more on the GSH redox cycle than on GSH de novo synthesis. The high hamster glutathione reductase (GRd) activity maintains high levels of GSH, which in turn, determine a low level of synthesis by feedback inhibition. γ-GCS, γ-glutamylcysteine synthetase (γ-glutamylcysteine ligaase; GPx, glutathione peroxidase; GSSG, oxidized glutathione; γ-Glu Cys, γ-glutamylcysteine; Gly, glycine; prot, protein. Right: on the other hand, in the rat, GSSG reduction is less prominent, and part of the larger production of GSSG is exported, leaving lower levels of GSH that determine less feedback inhibition on the synthesis. Thus in the rat, the steady-state concentration of reduced GSH seems to depend more on de novo GSH synthesis than on redox cycling.

in total, reduced, or oxidized glutathione content was observed (Fig. 2, B, D, and F).

**Time Course of Changes in Enzyme Activity and mRNA Expression**

γ-GCS. Figure 3A shows that in hamsters 4 h after elastase, γGCS activity remained within control values. The 60% reduction in the enzyme activity observed at 12 h (P < 0.01), recovered to basal values at 24 to 96 h. The significant reduction in γ-GCS activity observed 12 h after elastase instillation was coincident with the maximal depletion in total glutathione content (shown in Fig. 2A), whereas recovery of enzyme activity preceded total glutathione content recovery.

In contrast, rat lung γ-GCS activity was not affected by the procedure (Fig. 3B). A fourfold increase in γ-GCS mRNA expression was observed in hamsters 24 h after elastase, at the time enzyme activity started to recover (Fig. 3C), whereas no significant change in expression was observed in rats (Fig. 3D).

**GSH Redox Cycle Enzymes**

GPx. Fig. 4A shows that GPx activity in hamsters was reduced by 30% 4 h after elastase instillation (P < 0.01) and reached full recovery within a week. In contrast, in rats, a 20–25% transient reduction in enzyme activity fully recovered by 24 h (Fig. 4B). A sevenfold increase in GPx mRNA expression was detected in hamsters 24 h after elastase (Fig. 4C), whereas no change in expression was seen in rats (Fig. 4D).

GRd. This enzyme experienced the largest fall in activity after elastase instillation in the hamster. As early as 4 h, there was a 75% fall in its activity (P < 0.001), which remained low until reaching control values by day 4 (Fig. 5C). In the rat, GRd activity showed a transient 20–25% reduction 4 h after elastase (P < 0.05), returning to basal level by 12 h (Fig. 5D).

A fourfold increase in GRd mRNA expression was seen in hamsters 24 h after elastase, prior to the recovery in enzyme activity. Increased expression was seen also 4 days after elastase (Fig. 5C). In contrast, no change in expression was seen in rats (Fig. 5D).

**Recovery of Glutathione Homeostasis in the Lung of Hamsters After IT Elastase**

Most of the changes in glutathione content and in the activity of glutathione-related enzymes returned to control values at the end of the first week. We thought that by analyzing the recovery rate of GSH content and of the enzyme activities, insights could be obtained into the mechanisms involved in restoration of GSH levels.

A significant positive correlation was found between total glutathione content and γ-GCS activity (r = 0.38, P = 0.024) during the period of time in which lung GSH content recovered to reach baseline values (Fig. 6C). In contrast, no significant correlation was found between total glutathione content and either GPx or GRd activities during this period of time, suggesting that GSH level restoration might depend more on the synthesis of new GSH than on the redox cycle enzyme activities.

Data on total GSH content during the recovery period followed a best-fit (r = 0.98, P = 0.019) logarithmic curve (Fig. 6A, Eq. 3):

\[ \text{Total GSH} \times \% \text{ of control} = 66.8 + 3.03 \times \ln(t + 0.5) \quad (3) \]

where \( t \) is time in hours, and 66.8, 3.03, and 0.5 are constants.

Concordantly, γ-GCS activity was restored back to baseline, following a similar best-fit (r = 0.97, P = 0.028) logarithmic curve (Fig. 6B, Eq. 4):

\[ \gamma\text{-GCS} \times \% \text{ of control} = 15.9 + 16.4 \times \ln(t + 0.5) \quad (4) \]

where \( t \) is time in hours, and 15.9, 16.4, and 0.5 are constants.

**Markers of Severity of Elastase-Induced Acute Lung Injury**

Hamsters and rats differed in the type of lung response after treatment with elastase. Table 3 shows significant differences in markers of hemorrhage and of increased alveolar-capillary
permeability to proteins between hamsters and rats. Hamsters exhibited a large increment in BALF hemoglobin content in the first hours after elastase, reaching 7.5 (P < 0.01) and 5.7 (P < 0.05) times the control value at 4 and 24 h, respectively. In rats, the increment in BALF hemoglobin content was of a lower magnitude and occurred at a later time (only 2.8 times the control value at 24 h, P < 0.05).

Instead, rats showed evidence of a large increase in permeability 24 h after elastase, with almost a 10-fold increase in BALF total protein content (P < 0.001) and a reduction in the lung dry weight-to-wet weight ratio (P < 0.05). In hamsters, the increase in alveolar-capillary permeability was modest, with less than a twofold increase in BALF total protein content at 24 h (P < 0.05) and no significant change in the dry weight-to-wet weight ratio.

Species Differences in BALF α1-AT Electrophoretic Pattern

Figure 7, A and B shows representative α1-AT Western blots of BALF samples from rats and hamsters. Samples of BALF were obtained from control and elastase-treated animals at 4 and 24 h and at 4 and 7 days after instillation. In both species,
total α1-AT immunoreactivity increased after elastase treatment and returned to near control level at 7 days. This increase in α1-AT immunoreactivity related to an increment in alveolar capillary permeability to proteins, reflected in the Coomassie Blue stained gels of Fig. 7, C and D.

In rats (Fig. 7A), both control and 7-day BALF samples showed two α1-AT immunoreactive bands in the range of 52–60 kDa, corresponding to the native α1-AT described in rodents (23). As a result of elastase instillation, the two-band pattern for the native α1-AT became more evident and, two new bands were observed. First, a strong 88-kDa band, the molecular size of the [elastase+α1-AT] complex, appeared at 4 h and further increased at 24 h, to disappear at later time lapses. Second, a low molecular-size band of 48-kDa was found in most gels in this species either at 4 and/or 24 h, likely corresponding to the proteolytic fragment derived from the [elastase + α1-AT] interaction (41).

In hamsters (Fig. 7B), control and 7-day BALF samples showed almost undetectable levels of α1-AT immunoreactivity. As a result of elastase instillation, immunoreactivity in the 52 to 60 kDa range increased from 4 h to 24 h, suggesting the formation of the [elastase+α1-AT] complex, as observed in rats. However, unlike rats, a low molecular-size band was not clearly observed at 48-kDa in hamsters, likely due to the slower progression of the proteolytic process or lower activity levels.
60 kDa, corresponding to the native α1-AT exhibited either one or two bands. In this species, the 88-kDa band corresponding to the [elastase+α1-AT] complex and the 48-kDa band corresponding to the proteolytic fragment were not observed. These results were confirmed in gels with equivalent protein loading.

Figure 7, C and D compares at each time point, in Coomassie Blue-stained gels, the total protein profile of both serum and BALF samples obtained from a single representative animal, in an attempt to illustrate the effect of the increase in alveolar-capillary permeability induced by elastase on α1-AT levels in BALF.

In the control lane of the rat gel (Fig. 7C) there was a paucity of high molecular-size proteins in BALF, whereas 24 h after elastase treatment, BALF samples revealed the full spectrum of serum proteins, reflecting the loss in the size-selective barrier function of the alveolar capillary membrane, which was later restored 7 days after elastase instillation.

In hamsters, on the other hand (Fig. 7D), there was only a small increase in BALF total protein content after elastase treatment (see also data on Table 3) with mainly small molecular-size proteins contributing to total protein content. Thus, at 24 h, BALF protein profile was significantly different from the matched serum protein profile. These results suggest that there are species differences in alveolar capillary permeability changes after elastase treatment influencing BALF α1-AT levels differently.

In addition, whereas the rat showed evidence of elastase-α1-AT complex formation (88-kDa and 48-kDa bands) in hamsters that evidence was not present, suggesting functional inhibition of α1-AT.

Fig. 6. Recovery of glutathione homeostasis in the lungs of hamsters after intratracheal (IT) elastase. A: time course of total GSH content during the period in which GSH returns to baseline values. B: time course of γ-GCS activity during the period in which it returns to baseline values. C: relationship between total GSH content and γ-GCS activity during the phase of restoration of GSH homeostasis. Black symbols represent means ± 1 SE; lines correspond to the best-fit curves.
DISCUSSION

Our results showed significant interspecies differences in lung glutathione metabolism between Syrian Golden hamsters and Sprague-Dawley rats both at baseline and in the stage of diffuse alveolar damage induced by the same dose/100 g of body wt of IT elastase. Whereas the hamster lung, highly susceptible to injury by elastase, showed early total glutathione depletion and significant inhibition of the main enzymes involved in its metabolism, the less susceptible rat lung exhibited subtle or no reduction in GSH content or in GSH-related enzyme activities after elastase instillation. Homeostasis of GSH in the hamster lung was restored within 7 to 15 days, involving increased expression of the three enzymes studied, whereas no change in expression was found in rats. This early disruption in GSH metabolism renders the hamster lung more vulnerable to functional inhibition of α1-AT by inflammatory cell-derived oxidants (9, 10, 11, 20, 26) and thus, more susceptible to

<table>
<thead>
<tr>
<th>Table 3. Species differences in markers of acute lung injury</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>------------------------</td>
</tr>
<tr>
<td>Increment in BALF total protein, times over control</td>
</tr>
<tr>
<td>Increment in BALF hemoglobin, times over control</td>
</tr>
<tr>
<td>Lung, dry wt-to-wet wt ratio</td>
</tr>
</tbody>
</table>

BALF, bronchoalveolar lavage fluid. *P < 0.05, †P < 0.01.

Fig. 7. Western blot analysis for bronchoalveolar lavage fluid (BALF) α1-AT from rats (A) and hamsters (B) at different time points after elastase treatment compared with control BALF samples. In the rat blot (A) there was an increase in native α1-antitrypsin (α1-AT; 52–60 kDa) up to 24 h with a 88-kDa band and a 48-kDa band, likely corresponding, respectively, to the [elastase + α1-AT] complex and the proteolytic fragment derived after complex formation. In the hamster blot (B) these last two bands were not present, suggesting no [elastase + α1-AT] complex formation. C and D: correspond to Coomassie Blue stained gels showing the time course of changes in BALF protein profile after elastase treatment compared with serum samples. Control lanes show that the BALF protein profile significantly differed from the serum profile in both species. As a result of elastase instillation, BALF total protein content largely increased in rats and barely in hamsters. Thus, BALF protein profile became similar to that in the serum at 24 h in rats, whereas in hamster, the 24-h BALF protein profile did not resemble that in the serum, showing large differences in alveolar capillary permeability among the two species.
elastase than it would be considering only its low α₁-AT serum level (5, 19).

Elastase-Induced Lung Injury Is Not Associated with Upregulation of GSH-Related Enzyme Activity in Any Species

Since persistent mild chronic inflammation has been postulated as one of the mechanisms involved in progression of emphysema in this model, we had expected to find an upregulation of GSH-related enzyme activity in response to the inflammatory cell-derived chronic oxidant burden, similar to what has been shown by others with cigarette smoke (14, 17, 27, 42). Instead, after the early derangement in GSH metabolism during the stage of diffuse alveolar damage in hamsters, no further changes were observed, suggesting that GSH only plays a role in the early inflammatory stages of elastase-induced lung injury. In other words, in our model, the mild chronic inflammatory cell infiltrate that persists (4) and likely contributes to the progression of emphysema, seems not enough to represent a significant stimulus for the upregulation of enzymes involved in GSH metabolism.

Disruption in GSH Metabolism in the Hamster Lung After IT Instillation of Elastase

The main finding was a drop in GSH content without an increase in GSSG. If the glutathione depletion observed in the hamster lung was related to inflammatory cell-derived oxidative stress, we would have expected an increment in GSSG content (32), particularly since GRd activity was severely impaired for several days. Unexpectedly, GSSG content decreased significantly in the first hours after elastase, with a time course similar to that of total GSH. This pattern of GSH and GSSG behavior has not been described in another model of lung injury, except for the model of IT instillation of cigarette smoke condensate described by Rahman et al. in rats (31) and attributed by the investigators to GSH conjugate formation and transient inhibition of glutathione synthesis (31).

Interestingly, a similar pattern of GSH and GSSG depletion has been reported in the bronchoalveolar lavage fluid of human subjects after an acute episode of farmer’s lung disease induced experimentally by hay exposure under controlled and standardized conditions (3).

A common underlying feature in our model and in the two conditions mentioned above, is the acute inflammatory response involving the release of mediators with potential effects on GSH metabolism. In this regard, it has been shown in mice that IT instillation of elastase mediates the release of several growth factors from the lung in vivo (8). One of them is transforming growth factor-β1 (TGF-β1), which is released into bronchoalveolar lavage fluid in a time-dependent manner, reaching maximal levels 1–2 h after elastase (8). TGF-β1 is a key regulator of tissue repair mechanisms (22) with an effect on GSH synthesis since it downregulates the expression of γ-GCS (22). By this mechanism, it is thought that TGF-β1 increases the cytotoxicity to H₂O₂ in the human alveolar epithelial cell line A549 (1). Additionally, it has been shown that TGF-β1 inactivates γ-GCS in hepatocytes by protein cleavage induced by caspase activation (13). Thus, we speculate that mechanisms involving TGF-β1 release might participate, at least in part, in the aforementioned disruption of GSH metabolism in the hamster lung after IT elastase instillation.

Alternatively, GSH loss could be explained by mechanisms not related to its antioxidant function. It is known that GSH has many other functions in inflammatory reactions. As an example, it participates in the formation of the very potent proinflammatory mediators cysteine leukotrienes (12). Thus, one could hypothesize that species differences in lung GSH-related formation of cysteine leukotrienes after elastase instillation could explain part of the observed differences in lung GSH content.

The drop in total GSH content was associated with a significant reduction in the activity of the GSH-related enzymes. Our results showed that the two enzymes of the GSH redox cycle, that are crucial in maintaining intracellular GSH/GSSG homeostasis showed a significant reduction in activity in the first hours after elastase instillation, preceding the reduction in γ-GCS activity. Both the magnitude of the reduction in enzyme activity and the recovery rate were found to be enzyme-specific. The mechanisms by which the activity of GSH-related enzymes is reduced in the hamster lung early after IT elastase are not understood and require further study. Nevertheless, it is possible to speculate that TGF-β1-related effects, such as those already mentioned, could be involved in the reduction in γ-GCS activity. In addition, the three enzymes studied have been shown to be susceptible to oxidative inactivation by free radical species (2, 18, 42). Since these enzymes differ in the amount and type of amino acids, which are susceptible to oxidation, differences in the profile of their transient inactivation could be related to differences in oxidative modification of essential amino acid residues.

Restoration of GSH Levels to Basal Values After Elastase-Induced GSH Depletion

Our study showed that restoration of intracellular GSH level in the hamster lung involved increased expression of the three enzymes studied, preceding the recovery in activity. The increase in GSH synthesis seems to be the most important mechanism involved since on one hand, γ-GCS expression and activity increased prior to the increment in glutathione content and, on the other hand, γ-GCS activity significantly correlated with GSH content during the recovery period. In this context, an increase in γ-GCS activity due to enzyme neo-synthesis and posttransductional mechanisms has been found by others to be associated with restoration of glutathione levels in lung cells and tissue after oxidative stress in several experimental conditions (32, 36). With regard to the enzymes of the redox cycle, they could also play a role since their expression was also transiently increased; however, no correlation was found by us between glutathione content and redox cycle enzyme activities during the recovery period.

Functional Inhibition of α₁-AT in Relation to the Derangement in GSH Metabolism in Hamsters

The study of BALF showing that hamsters do not have the elastase-α₁-AT complex formation found in rats, provides evidence of functional inhibition of α₁-AT in the first hours after elastase treatment, in association with changes in GSH and related enzyme systems. Thus, the hamster is more sus-
ceptible to injury than it would be considering only its low α1-AT level.

Species Differences in Basal Lung GSH Metabolism Contributing to GSH Response After Elastase Treatment

Variations in basal lung glutathione-related antioxidant capacity between hamsters and rats that could contribute to species differences in GSH modifications after elastase have not been systematically studied. We could find only one study comparing lung antioxidant enzymes between rats and hamsters that included at least one GSH-related enzyme. In that study, Bryan and Jenkinson (7), in agreement with our finding, showed that the rat lung has a higher GPx activity than the hamster lung.

In our study, although basal γ-GCS and GPx activities were lower in the elastase-sensitive species, GRd activity was actually higher. The way we interpret our results is that steady-state GSH concentration in the hamster lung might depend more on the GSH redox cycle than on GSH synthesis. The high GRd activity maintains high levels of GSH, which by feedback inhibition, determine a low level of synthesis. Thus the hamster lung could be considered as a GSH recycler. The opposite might be true for the elastase-resistant rat lung in which steady-state concentration of GSH seems to depend more on GSH synthesis than on redox cycling (Table 2 and Fig. 1).

Elastase treatment in the hamster, although affecting the three GSH-related enzymes, had a larger (75% inhibition) and longer-lasting effect on GRd, precisely the main enzyme for GSH homeostasis in this animal. In our view, this is an example of species differences in basal GSH metabolism playing a role in species differences in GSH response to injurious agents.

Lack of Disruption of Glutathione Metabolism in the Rat Lung After IT Elastase Instillation

At this point, we cannot exclude the possibility that differences in GSH metabolism after elastase instillation among hamsters and rats could be the result of species differences in the magnitude of elastase-induced diffuse alveolar damage. As a matter of fact, markers of acute lung injury were significantly different among hamsters and rats. Whereas rats showed a large increase in alveolar-capillary permeability with little hemorrhagic changes, hamsters exhibited significant, early hemorrhagic changes, suggesting the possibility of more severe acute lung injury in this animal. In this context, the lack of an effect on rat lung GSH metabolism after IT elastase instillation could only be the reflection of a less severe diffuse alveolar damage compared with the hamster. We propose that the high serum and lung α1-AT levels known to be present in the rat compared with the hamster (5, 19) could limit the elastolytic attack to matrix components resulting in less TGF-β1-dependent effects on glutathione metabolism.

Notwithstanding the higher serum and lung level of α1-AT (5, 19), the maintenance of redox status in the rat lung during the stage of elastase-induced alveolar damage, contributes to preserve antiprotease function.

In conclusion, we have found significant species differences in GSH metabolism early after IT elastase instillation and an association between disruption in glutathione metabolism and more severe chronic lung damage in the hamster. Oxidant/antioxidant imbalance resulting from the disrupted glutathione metabolism in the first hours of elastase-induced acute lung injury in the hamster is associated with less functional α1-AT that contributes to worsen elastase-induced protease-antiprotease imbalance, favoring a more severe emphysema development, since it has been shown that events that occur in the first hours or days after elastase instillation, largely determine the magnitude of permanent emphysema development (25).

Perspectives and Significance

In the elastase model of emphysema, the hamster vulnerability to emphysema development relates to a derangement in the steady-state concentration of antioxidants that favors functional inhibition of the already low levels of α1-AT. The study suggests that antioxidant lung defenses are very important under conditions of low levels of antiproteases, a notion also applicable to deficiency of α1-AT in humans. Further studies using antioxidants could reduce disease severity in this model.

ACKNOWLEDGMENTS

The authors thank Oriana Urrutia for her assistance with enzymatic analyses.

GRANTS

This study was supported by National Fund for Scientific and Technological Development (FONDECYT) Grants 1030412 and 11070121.

REFERENCES
