Decreased glutathione content and glutathione S-transferase activity in red blood cells of coal miners with early stages of pneumoconiosis

Chris T A Evelo, Robert P Bos, Paul J A Borm

Abstract
Blood samples of miners heavily exposed to coal dust were examined for changes in glutathione S-transferase (GST) activity. Decreased GST activity was found in red blood cells of subjects with early stages of coal workers' pneumoconiosis (International Labour Office classification 0/1–1/2) when compared with control miners. At further progression of coal workers' pneumoconiosis (≥2/1), the activity of GST was not different from controls. In the same group with moderate coal workers’ pneumoconiosis, a decrease in GSH in red blood cells occurred. Decreases in GST activity in early stages of coal workers’ pneumoconiosis, as well as the decreases in glutathione peroxidase (GPx) activity and in GSH concentrations reported earlier, may originate from damage caused by reactive oxygen species. These changes might imply an impairment of the detoxification capacity for electrophilic and oxidative compounds during this stage of the disease.

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Lung fibrosis related to the inhalation of dust containing toxic particles such as silica or asbestos is a serious occupational hazard. It is well known that during the immune defence against dust particles, reactive oxidant species are secreted by various types of inflammatory cells with alveolar macrophages playing a key part in this process. The lung tissue possesses an elaborate defence mechanism to detoxify reactive oxidant species, constituted by a set of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (GPx) and low-molecular weight compounds such as glutathione (GSH) acting as a substrate for GPx and glutathione S-transferase (GST) or as direct radical scavengers. Also, antioxidants localised in erythrocytes are involved in the antioxidant protection of the lung, acting as circulating antioxidant carriers.

Previously, we showed that antioxidant enzymes and GSH in erythrocytes are affected at different stages of pneumoconiosis in coal workers or silicotic patients and in lung tissue of rats exposed to silica; GSH concentration and GST activity were also found to be affected after occupational exposure to reactive compounds and in situations involving reactive oxidant species stress such as smoking and heavy exercise. This paper presents the results of GSH determinations and GST analyses with an activity assay and enzyme linked immunosorbent assay (ELISA) in red blood cells of a cohort of coal miners previously investigated by our research group.

Materials and methods
SUBJECTS
Ninety one coal miners, all heavily exposed to coal dust underground at the coal face for at least 12 years, were selected from the Belgian coal mining industry pits (Kempense Steenkolenmijnen). Blood samples were taken and treated as described previously. Control miners (n = 58) and miners with coal workers’ pneumoconiosis (n = 33) were matched for exposure and age at which they started work. In this study persons with the classification 0/1, 1/0, 1/1, and 1/2 were gathered in group 2 (n = 19); persons with classification 2/1 and higher in group 3 (n = 14); control miners constituted group 1.

ANALYTICAL METHODS
Chemicals
The chemicals used were: rabbit polyclonal antibodies against GSTp (Medlabs; Dublin, Ireland), swine anti-rabbit IgG antibodies conjugated to horse radish peroxidase and orthophenylenediamine (OPD) (Dakopatts; Copenhagen, Denmark), 5,5'-dithiobis-(2-nitrobenzoic acid) (EODA).
Merck; Darmstadt, Germany). Reduced glutathione (Boehringer Mannheim; Mannheim, Germany), 1-chloro 2,4-dinitrobenzene (CDNB), dithiotreitol, GST\(_\pi\) from human placenta, and tween 20 (Sigma; St Louis, USA). All other chemicals were of analytical quality. Only microfiltered deionised water was used.

**Determination of blood GSH**

Total GSH concentration was determined with the cyclic oxidation reduction method essentially as described by Anderson.\(^{13}\)

**Assays for GST**

Activity of GST (EC 2.5.1.18) was determined by a modification\(^{11}\) of the method described by Habig and Jacoby,\(^ {14}\) lysing the erythrocytes by addition of three volumes of water containing 1.4 mmol l\(^{-1}\) neutral DTT, centrifugation, and measurement of the activity towards CDNB. A Cary 118 spectrophotometer was used in auto slit mode with the gain adjusted to 2-5.

For the competitive ELISA, Costar flat bottom 96 well EIA plates (Data Packaging Corporation; Broadway, MA, USA) were coated overnight at 4°C with 200 \(\mu\)l phosphate buffered (10 mmol l\(^{-1}\)) saline of pH 7.2 containing 11 \(\mu\)L GST\(_\pi\) from human placenta per well. Each well was washed three times with 200 \(\mu\)L ELISA buffer (10 mmol l\(^{-1}\) sodium phosphate with 0.5 mol l\(^{-1}\) sodium chloride and 0.1% (v/v) tween-20) of pH 7-2. This and all further steps were performed at room temperature. To each well 50 \(\mu\)L of the antigen solution (haemolysate) and 150 \(\mu\)L antibodies against GST\(_\pi\) diluted 2250-fold with ELISA buffer were added. After two hours of incubation the wells were washed three times with ELISA buffer and incubated for one hour with 200 \(\mu\)L horse radish peroxidase labelled anti-IgG diluted 1500-fold with ELISA buffer. After another three washes with ELISA buffer 100 \(\mu\)L of a 100 mmol l\(^{-1}\) citrate-phosphate buffer (pH 5.0) containing 0.67 mg/ml OPD and 0.0125% (w/v) \(\text{H}_2\text{O}_2\) were added to each well. After exactly 15 minutes the enzymatic reaction was stopped by addition of 150 \(\mu\)L 1 M sulphuric acid. The extinctions at 492 nm were read with a multispan interfaced to a MINC computer.

Reference data for the quantification of the ELISA results were obtained with GST\(_\pi\) from human placenta. Known quantities of this enzyme—expressed in activity units—were tested in the competitive ELISA assay. The results were fitted to the general logistic function:\(^{15}\)

\[
Y = \frac{a - d}{1 + (\frac{C}{EC50})^s} + d
\]

where \(Y\) is the response; \(C\) the concentration of the antigen; \(a\) the response when \(C = 0\); \(d\) the response for "infinite" concentrations of antigen; \(EC50\) the antigen concentration resulting in a response half way between \(a\) and \(d\); and \(s\) is a slope factor that corresponds to the slope of the logit-log plot, when \(C\) is plotted in terms of natural logarithms. For curve fitting the NLIN procedure from the SAS package was used with the DUD method for non-linear regression.\(^ {16}\) The response was expressed as a fraction of the maximum extinction, found without addition of antigen, \(E = Y/a\).

The haemoglobin concentrations in the erythrocytes were determined with the haemoglobin cyanide procedure.\(^ {17}\)

**Statistical Evaluations**

Analytical results were coupled to previous data on this cohort in a database. Correlations between variables were studied using STATGRAPH 3.01 statistical package. Differences between variables in separate groups were evaluated with the Wilcoxon 2 sample test.

**Results**

The table shows the mean values and standard deviations (SDs) of GSH concentrations, GST activity towards CDNB and GST content as determined by competitive ELISA in the blood of the control miners and the two groups of miners with coal workers’ pneumoconiosis. No effect of smoking was seen on any variable mentioned. Concentrations of GSH were determined previously.\(^ {1}\) Repeated analyses of the frozen samples used for the GST determinations did not show significant differences from the original values. Therefore, the original data rearranged for the current purpose are listed in the table. Both GSH concentration and GST activity were decreased in group 2, the workers with moderate forms of coal workers’ pneumoconiosis. In group 3 such a decrease was not found. The GST protein content showed a tendency to follow the GST activities, although the decrease in group 2 did not attain statistical significance.

**Discussion**

Our data show that, as well as GSH, erythrocyte GST activity is decreased in subjects with early stages of pulmonary fibrosis (0/1 – 1/2) when compared with control miners. At further progression of coal workers’ pneumoconiosis (2/1+), however, erythrocyte activity was not different from controls. In the same group with moderate coal workers’ pneumoconiosis, a decrease in GSH in erythrocytes occurs. Previously, we reported an increase in total GPx activity in the same workers at early stages of the disease\(^ {1}\) and in rat lung homogenates after in vivo exposure to silica or...
Glutathione (GSH) concentrations, glutathione S-transferase (GST) activity towards CDNB, and glutathione S-transferase protein content determined by ELISA in red blood cells of coal workers

<table>
<thead>
<tr>
<th>Control Group (0/0) Mean (SD)</th>
<th>Group 2 CWP (0/1–1/2) Mean (SD)</th>
<th>Group 3 CWP (&gt;2/1) Mean (SD)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSH (mmol·g·Hb&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>3.97 (0.08)</td>
<td>3.62 (0.14)</td>
<td>3.98 (0.46)</td>
</tr>
<tr>
<td>n = 58</td>
<td>n = 19</td>
<td>n = 14</td>
<td></td>
</tr>
<tr>
<td>GST (CDNB)</td>
<td>2.99 (1.36)</td>
<td>2.31 (1.09)</td>
<td>3.37 (1.14)</td>
</tr>
<tr>
<td>(U·g·Hb&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>n = 45</td>
<td>n = 9</td>
<td>n = 10</td>
</tr>
<tr>
<td>GST (ELISA)</td>
<td>2.46 (1.93)</td>
<td>1.92 (0.90)</td>
<td>2.54 (1.39)</td>
</tr>
<tr>
<td>(equiv U·g·Hb&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>n = 34</td>
<td>n = 6</td>
<td>n = 7</td>
</tr>
</tbody>
</table>

p Values are for group 2 v control miners (Wilcoxon 2 sample test).

We think that the decrease in GSH concentration and GST activity is caused by excessive release of reactive oxidant species by alveolar macrophages and neutrophils in the lung tissue. A decrease in GST activity cannot be the result of decreased GSH concentrations during determinations, as saturating concentrations of GSH (1 mmol·l<sup>−1</sup>) are added in the assay. Moreover, there is no (negative) correlation between GSH content and GST activity of the blood samples. Combined with our data obtained with the ELISA on GST in erythrocytes (which tended to decrease in the same group) we conclude that a fraction of GST is damaged, possibly by oxidation of SH sites. The decrease in available GST activity plus the fact that GST activity is GSH dependent, might imply that in vivo functioning of the enzyme is affected rendering these subjects more susceptible to damage resulting from lipid peroxidation or from coexposure to electrophilic compounds. Various mineral fibres were reported to inhibit GST activity in the cytosol of rat lung homogenates after in vitro and in vivo exposure, whereas phase 1 reactions were increased at advanced stages of the disease. An induction of phase I enzymes in advanced stages of fibrosis might further aggregate the accumulation of activated metabolites in the lung.

Previously, we argued that the restoration of erythrocyte GST activity to control values in miners with coal workers’ pneumoconiosis beyond 2/1 is caused by a hepatic efflux of GSH. We found comparable phenomena for GST in previously untrained subjects. After moderate running training (up to 60 minutes after 20 weeks) GST activity and GSH concentrations in erythrocytes were both increased. After heavy training (up to 90 minutes after 40 weeks) GSH values returned to normal and GST was substantially increased. Increases in GST protein content in erythrocytes can be the result of increased protein synthesis during erythrocyte proliferation, or, less likely, of increased longevity of the protein itself. Increases in GST content were also found in smokers. Here lower specific activities and equal total activities were found. This too might be a result of compensatory GST synthesis. Thus GST activity in coal miners with coal miners’ pneumoconiosis >2/1 could remain at normal values due to compensation of inactivation by extra protein synthesis during erythrocyte proliferation. This extra synthesis could be triggered by inflammatory processes generating cytokine release in coal workers’ pneumoconiosis or heavy exercise. Possibly the formation of lipid peroxides, reported to occur during exercise and in rats exposed to mineral fibres, could be an important factor in this respect. Lipid peroxides are known to be detoxified by GST, which shows selenium independent GPx activity.

In conclusion, GST activity in erythrocytes is affected in early phases of coal workers’ pneumoconiosis, possibly as a result of oxidative damage. Combined with our earlier findings (a decrease in GSH concentration and an increase of GPx activity), this might mean that detoxification of electrophilic compounds in these stages is impaired.

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