A Correlation between Glutathione Levels and Cellular Damage in Isolated Hepatocytes

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To obtain high levels of glutathione in isolated hepatocytes an isolation procedure shorter than 16 min was used. This procedure gave a moderately high yield of viable cells (200 – 300 × 10⁶ cells/10 g liver) with 44 ± 3 nmol of glutathione/10⁶ cells. Incubation in Krebs-Henseleit solution containing 2% albumin resulted in a continuous loss of reduced glutathione from the cells, while incubation in a medium containing amino acids and horse serum resulted in increased levels, suggesting active synthesis for 5 h. A short and apparently harmless depletion of reduced glutathione was induced by diethylmaleate or cumene hydroperoxide. A depletion of reduced glutathione lasting more than 1 h was accompanied by an increased cellular leakage. The depletion was induced by either diethylmaleate plus paracetamol or diethylmaleate alone in higher concentrations. A common mechanism for these toxic responses is suggested.

Glutathione is a thiol that has been implicated in the maintenance of cellular integrity and decreased levels of glutathione in the liver may be either directly or indirectly responsible for cellular damage. Of physiological importance may be the regulation of enzyme function [1] and protein synthesis [2]. Furthermore glutathione is a substrate for glutathione-peroxidase and may be a significant factor in minimizing the rate of cellular peroxidation [3]. Studies in this laboratory on isolated hepatocytes substantiated this suggestion [4 – 7].

The toxic effect of the analgesic drug paracetamol (acetaminophen) illustrates a possible relationship between drug-induced liver damage and glutathione metabolism [8 – 11]. Under normal conditions glutathione forms conjugates with oxygenated metabolites of this drug. However, in the absence of glutathione alkylation of macromolecules occurs, which has been interpreted as the direct cause for cell damage.

Glutathione conjugates of foreign compounds or their metabolites may be excreted from the liver through the plasma membrane since they are more water-soluble than the parent compound [12]. Glutathione may also be excreted as the oxidized disulfide, which has been observed in perfused liver systems [13] and isolated hepatocytes [5]. A related mechanism for glutathione turnover in erythrocytes has been discussed [14].

It has been observed in this laboratory that with different preparations of isolated hepatocytes the levels of glutathione varied considerably, notably with levels far below those anticipated in the liver cell in situ. Furthermore, a continuous loss of reduced glutathione occurred during incubation [5]. Thus, it seemed plausible that precautions to maintain levels of reduced and oxidized glutathione might ensure cell viability in a suspension of isolated hepatocytes. Accordingly, the purpose of this study was to characterize the factors necessary to preserve glutathione levels in the isolated hepatocyte. Without knowledge of these factors the use of isolated hepatocytes in toxicological studies might be of limited value, particularly in those instances in which glutathione conjugation is an obligatory reaction in the metabolism of the toxic agent under study.

MATERIALS AND METHODS

Isolated hepatocytes were prepared from male Sprague-Dawley rats, weighing between 210 – 230 g, that had free access to food and water. The isolation
Fig. 1. Diagram of the perfusion system. The liver was immersed, suspended from the tied cannula, in the perfusate in the thermostated reservoir (37°C). The top opening of the oxygenator served as a shunt to bypass the liver, and transported the gas to the reservoir. A plastic screen at the bottom of the reservoir filtered the fluid before it was recirculated. The flow through the cannula was adjustable from a few drops to 100 ml per min by changing the gas flow and/or the capacity of the shunt. The dead volume (in the oxygenator and the tubings) was 40 ml.

procedure is basically the same as that described in earlier publications [4,5]. Some modifications have been introduced. The perfusion system is described in Fig.1 and was designed with a low dead volume, ease of cleaning and sterilization and adjustable flow rates within wide limits while maintaining constant pressure in the system. The pressure was kept at 10–15 cm H₂O and the flow rate adjusted (to about 30 ml/min) so that the liver resumed its normal shape. The perfusion time was 4 min with a modified Hank’s solution containing 2% albumin and EGTA (0.5 mM) ("solution A") and 6 min with a modified Hanks’ solution containing 0.12% collagenase and Ca²⁺ (4 mM) ("solution B"). At this time the liver was somewhat swollen. The liver capsule was gently disrupted with scissors and forceps and the cells dispersed by gentle agitation by hand in 75 ml Krebs-Henseleit solution containing 2% albumin ("salt-albumin solution") at 37°C. Residual connective tissue and undispersed cells were removed by filtration through gauze and the filtrate allowed to stand in a beaker (7 cm diameter) at ambient temperature while a stream of O₂ and CO₂ (95%:5%) was directed over the surface. A loose pellet formed within 2–3 min and the supernatant was removed by aspiration. The pellet was then immediately diluted 20-fold in the incubation solution containing 2% albumin ("salt-albumin solution") at 37°C. Residual connective tissue and undispersed cells were removed by filtration through gauze and the filtrate allowed to stand in a beaker (7 cm diameter) at ambient temperature while a stream of O₂ and CO₂ (95%:5%) was directed over the surface. A loose pellet formed within 2–3 min and the supernatant was removed by aspiration. The pellet was then immediately diluted 20-fold in the incubation solution (see below). 97–100% of the cells in the pellet excluded trypan blue and the yield of viable cells was between 200–300 x 10⁶ cells/liver. By electron microscopy, hepatocytes were of normal appearance (cf. [6]) and did not exhibit signs of anoxia.

To maintain a maximum number of viable cells during 5 h of incubation, experiments were performed in round-bottomed flasks (50 ml) fitted on a standard taper distillation adapter for 5 flasks (Büchi) which were rotated (30 rev./min) on a rotary evaporator. The incubate volume was 20 ml and contained 16–30 x 10⁶ cells. Gas was introduced via a plastic tubing inserted through the vacuum exit of the evaporator. The flasks were rotated through a thermostated water bath (37°C) at an axis of rotation of 45°C to the water surface. The solution used for incubation was either the "salt-albumin solution" or a "complete medium" (Waymouth MB 752/1 medium, Gibco Biocult Ltd, Scotland, +17.5% horse serum, inactivated for 30 min at 56°C + 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate buffer) (unless otherwise specified). In all experiments the medium contained heparin (10 I.U./ml) and penicillin (500 I.U./ml). The complete medium used was essentially the same as that described by Jeejeebhoy et al. [15].

To estimate the number of viable cells during incubation, lactate dehydrogenase activity was monitored periodically. The activity was measured in the total incubate before and after addition of detergent (sodium deoxycholate, Triton X-100) to estimate the latency of the enzyme. Pyruvate (0.67 mM final concentration) and NADH (0.1 mM final concentration) were included and the rate of NADH oxidation monitored at 340 nm [6]. It was found occasionally that the activity measured after lysis of the cells decreased with time (cf. Fig.6) suggesting proteolysis of lactate dehydrogenase in the incubate. Glutathione was measured with a colorimetric method for thiols [16] or with a fluorometric procedure specific for glutathione [17]. Both gave similar results and were used to measure reduced and oxidized glutathione in isolated cells. Measurements were performed on cells reharvested by gentle centrifugation (50 x g). Malondialdehyde was measured in the total incubate as described previously [4].

The results are mainly presented as "typical experiments", two or more of which were performed with separate batches of cells.
Table 1. Glutathione content in isolated hepatocytes

The total amount of glutathione (reduced + oxidized) was measured. In (1) and (3) the number of experiments is indicated in the parentheses. In (2) perfusate was not recirculated during excision of the liver. The excision took 2 min, after which the liver was placed in the perfusate reservoir (cf. Fig. 1), and the fluid recirculated for 13 or 28 min. The solutes were either a modified Locke’s solution containing 2% albumin and EGTA (0.5 mM) or “solution A”. The experimental results are from one experiment typical of three.

The recovery of glutathione in a typical isolation procedure (see Materials and Methods). The sedimented cells were diluted 20-fold in the “complete medium” before glutathione was determined.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Glutathione content per 10⁶ cells total</th>
<th>nmol</th>
<th>µmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cells isolated as described in [4,5]</td>
<td>30 ± 13 (10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Perfusates recirculated through the liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locke’s solution + EGTA; 13 min</td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Locke’s solution + EGTA; 28 min</td>
<td>6.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>“solution A” 13 min</td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Cells isolated by present method</td>
<td>44 ± 3 (5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Cells, debris and solutes recovered after isolation of cells by present method</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>“Solution A” (first perfusion; 2 min)</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>“Solution B” (second perfusion; 6 min)</td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver debris (undigested parts of the liver, damaged cells)</td>
<td>74.0</td>
<td></td>
<td></td>
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<tr>
<td>“Salt-albumin solution” (cell wash)</td>
<td>9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isolated hepatocytes (240 x 10⁶ cells)</td>
<td>10.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RESULTS

Approximately 6.1 ± 0.5 µmol of glutathione were found per gram wet weight liver, which is in good agreement with reported values [13]. Since 1 g of liver contains about 100 x 10⁶ cells [18], 10⁶ cells in situ should contain about 60 nmol of glutathione. The glutathione content of hepatocytes isolated according to previously described methods varied greatly but was always less than anticipated (Table 1). Prolonged perfusion did not lower the yield of viable hepatocytes, but resulted in a continuous release of glutathione, which tended to increase with time (Table 1). The simplified method described herein gave a lower yield of cells but the cells contained greater quantities of glutathione (Table 1).

During a five-hour incubation in the salt-albumin solution (Fig. 2) a linear decrease in reduced glutathione was seen. When the cells were incubated in the complete medium the amount of reduced glutathione increased (Fig. 2). The level of oxidized glutathione did not change significantly. An increase in total glutathione was evident when measured in the total incubate as well (data not presented). Cells with an initially low level of glutathione showed a similar increase as those with an initially high level (Fig. 3; cf. Fig. 2). The same effect was also seen when a rapid oxidation of glutathione had been induced (cf. [5]) by addition of cumene hydroperoxide (Fig. 3). Fig. 4 shows the glutathione content of isolated hepatocytes during incubation in various media. The Krebs-Henseleit salt solution supplemented with horse serum and amino acids gave results similar to the complete medium. Amino acids alone had no effect while horse serum supplementation maintained a constant glutathione level in the cells.

Paracetamol exerted its maximal effect on the reduced glutathione level at 10 mM concentration.
Glutathione and Cell Damage

Fig. 4. Glutathione content of isolated hepatocytes incubated in modified salt solutions. The different salt solutions were: Krebs-Henseleit's solution containing 2% albumin and a 25-fold diluted solution of amino acids (50-fold concentrated minimal essential (MEM) amino acid solution, Gibco Bio-cult 113 G) (x--x), Krebs-Henseleit's solution containing 17.5% horse serum (0-0) and Krebs-Henseleit's solution containing the 25-fold diluted amino acid solution plus 17.5% horse serum (○-○). The results are from an experiment typical of three experiments. (Fig. 2), while no effect was noted on the level of oxidized glutathione (results not presented). With drug concentrations below 10 mM the effect on reduced glutathione appeared to be related to the drug concentration; at 2.5 mM the glutathione levels were constant for 5 h (cf. Fig. 5). Diethylmaleate decreased glutathione levels markedly within 5 min. This effect was also seen in earlier studies [5]. The addition of diethylmaleate, in approximately equimolar amounts with reduced glutathione, decreased the glutathione content by more than 50% in these cells (Fig. 5). With 18 μmol of diethylmaleate or less per 20 ml incubate, a recovery of glutathione levels was seen after the first hour and no signs of cell damage were evident (Fig. 5 and 6). Two different treatments of the cells with diethylmaleate and/or paracetamol induced similar toxic effects, as evidenced by increased leakiness after 2 h. Diethylmaleate (36 μmol) was as toxic as one-half that amount combined with a low dose of paracetamol (2.5 mM) (Fig. 6). It was noted that upon such treatment the levels of glutathione was virtually zero, and that no recovery of glutathione levels occurred (Fig. 5). It was further seen that malondialdehyde, a split product of peroxidized lipids, accumulated in these incubates (data not presented).

DISCUSSION

The basic technique to isolated hepatocytes used in this study has been described with many modifications [15, 19-24] and yields the greatest degree of trypan blue exclusion [19]. However, these isolated cells probably contain less glutathione than average liver cells in situ. Possibly, the simplest way to minimize this loss is to shorten the time required for cell iso-
lation. The two perfusion times need not exceed 4 and 6 min, respectively, to achieve uniform results. By omitting dispersion of the cells in a second solution containing collagenase and by simplifying the washing procedure, time was further shortened and losses in glutathione minimized. Provided that the highest possible yield of cells are not a requisite, the isolation time need not exceed 15 min. Other modifications of the isolation technique, such as isolation in a complete incubation medium or in one supplemented with glutathione, may well result in greater yields of hepatocytes with high levels of glutathione, but these parameters have not been examined.

Excretion of oxidized glutathione may, at least partly, explain the decreased levels of glutathione; the marked oxidation, initiated by cumene hydroperoxide (Fig. 3), was never compensated for during the successive hours of incubation even though intracellular levels of oxidized glutathione decreased. Instead there was a striking parallelism during recovery between controls and peroxide-treated cells. This indicates that the reducing capacity of glutathione in the cells was not sufficient. Glutathione synthesis on the other hand, could compensate the observed losses, provided the cells were incubated in the complete medium. The prerequisites for glutathione synthesis in the isolated hepatocytes have not yet been well characterized. The concentration of one or several amino acid(s) in the medium seems critical for maximal glutathione synthesis while the role of horse serum is obscure. An understanding of this problem may be of clinical importance since maintaining glutathione synthesis is a goal in treating paracetamol-intoxicated patients.

The levels of glutathione were decreased by addition of diethylmaleate and paracetamol. Diethylmaleate effectively decreases the glutathione level in vivo [25], presumably by direct conjugation [12]. When isolated hepatocytes were depleted with diethylmaleate in moderate excess no signs of cell damage were elicited. These results together with those obtained with cumene hydroperoxide (Fig. 3) support the thesis that a sudden depletion of hepatic glutathione is harmless, provided that the conditions for resynthesis are favorable. Paracetamol, on the other hand, is first oxidized by a (cytochrome-P-450)-dependent step and then conjugated to glutathione in a second step [8–11]. It is well established that isolated hepatocytes actively oxidize drugs [26], and the consumption of glutathione may be taken as evidence for paracetamol metabolism. Cells isolated from phenobarbital-treated rats were depleted of glutathione within 6 h [27], while cells used in this study could not be depleted by paracetamol alone and no toxic effects were observed. However, the combined effect of diethylmaleate and paracetamol induced a state of long lasting depletion. An excessive dose of diethylmaleate produced the same effect. Both these treatments were followed by a similar toxic effect, evident after 2 h, suggesting similar mechanisms for cell damage. The observation that malondialdehyde accumulated suggests lipid peroxidation as a possible important factor in such a mechanism. Thus, it seems possible to throw some doubt on the theory that paracetamol toxicity is a direct function of alkylation.

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REFERENCES


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