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INCREASE IN HEPATIC MIXED DISULPHIDE AND GLUTATHIONE DISULPHIDE LEVELS ELICITED BY PARAQUAT*

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Abstract—Paraquat (1 mM), when added to isolated haemoglobin-free perfused rat liver, leads to an increase of intracellular mixed disulphides from 1.3 μmole GSH equivalents per g wet weight in the controls to 2.5 μmole/g. This raises the proportion of mixed disulphides to total glutathione equivalents from about 0.2 at the beginning of the perfusion to about 0.4. The mixed disulphides are predominantly protein-bound, with low molecular weight compounds being quantitatively negligible.

The content of intracellular glutathione disulphide (GSSG) is increased from 17 nmole/g in the controls to 38 nmole/g in the presence of paraquat. In addition, there is an increased rate of release of GSSG into the extracellular (biliary) space, reported previously.

It is suggested that, in a reaction catalysed by thioltransferase(s), the rise in GSSG is correlated with the rise in mixed disulphides (reaction 1). Occupancy of potential cellular mixed disulphide sites is about 1/2 in the controls, and rises to about 2/3 in the presence of paraquat.

The ratio of cellular contents, NADPH/$NADP^+$, is decreased from 5.1 in the controls to 2.3 in the presence of paraquat, while the sum of NADPH plus $NADP^+$ remains unaltered.

The perturbation in the glutathione status may be related to metabolic effects such as the stimulation of the pentose-phosphate pathway activity, and possibly also to the expression of toxic effects.

Treatment of mice with the herbicide paraquat (1,1′-dimethyl-4,4′-bipyridylium; methyl viologen) leads to a decrease of reduced glutathione (GSH) in the liver [1], as was also observed in rat livers perfused with paraquat [2]. The loss of GSH was found to be due, in part, to an increased release of oxidized glutathione (GSSG) into the bile, and it was further suggested that there is a mechanism in addition to GSSG efflux by which paraquat can affect the hepatic content of GSH [2].

We have examined here whether paraquat decreases the GSH content by the formation of mixed disulphides of proteins or low molecular weight thiol compounds. Mixed disulphides have been found in various tissues with amounts depending on age and diurnal or nutritional state [3–7], and possibly they are also of importance in expression of toxicity, for instance by perturbation of critical membrane functions. Mixed disulphides can be formed, for example, by reaction (1):

$$\text{prot–SH} + \text{GSSG} \rightleftharpoons \text{prot–SSG} + \text{GSH} \quad (1)$$

A thiol transferase catalysing reaction (1) has been isolated from cytosol of rat liver [8].

Recently, a linear relationship between the intracellular concentration of GSSG and the rate of its release into the bile was found [9]. If the paraquat-stimulated biliary GSSG-release [2] also is associated with increased intracellular GSSG concentrations, then the formation of mixed disulphides could be stimulated via reaction (1).

MATERIALS AND METHODS

Perfusion and sampling. Livers from male Wistar rats (170–200 g) were perfused in a haemoglobin-free non-recirculating system with Krebs–Henseleit bicarbonate buffer, pH 7.4, saturated with an oxygen/carbon dioxide mixture (95/5) [10]. In experiments with paraquat, after 20 min of initial perfusion the medium contained 1 mmole/l paraquat (Fluka, Neu-Ulm, West Germany) for a period of 120 min. At the times given in Figs. 1 and 2 an aliquot of the liver tissue (about 0.5 g) was cut off, immediately frozen in liquid nitrogen and pulverized. The frozen liver powder was used for estimation of GSH, total glutathione equivalents, mixed disulphides and GSSG (see also [11]). Experiments were started between 9 and 10 a.m., and assays were performed on the same day.

Acid soluble GSH. Liver powder (50 mg) was weighed into 3.75 ml 0.1 M Tris–HCl buffer, pH 8.0, +1 ml 25% metaphosphoric acid, homogenized for 20 sec in an Ultra Turrax and centrifuged for 15 min at 100,000 *g* in a SW 56 rotor (Beckman) [12]. Of the supernatant 0.05 ml was pipetted into 4.5 ml 0.1 M potassium phosphate (5 mM EDTA) buffer, pH 8.0. This was followed by a second dilution step to exclude interference of metaphosphoric acid and possible quenching compounds in the liver extract with the test system [13] by pipetting 0.1 ml of the first dilution into 1.8 ml phosphate buffer, and then 0.1 ml *o*-phthalaldehyde reagent (1 mg/ml in methanol) was added. Exactly after 15 min, fluorescence

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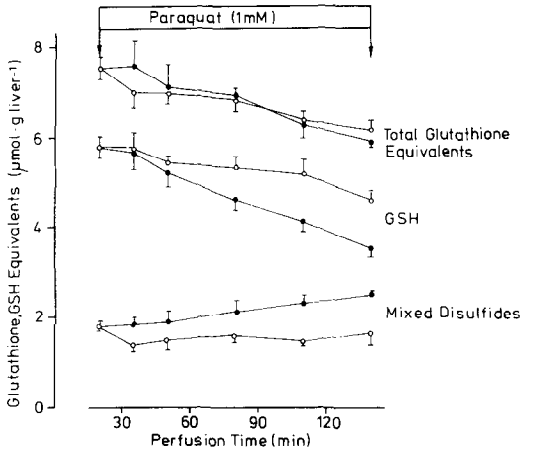

Fig. 1. Time course of intracellular levels of total glutathione equivalents, GSH and mixed disulphides in perfused rat livers. Control (○), and paraquat, 1 mM (●), added after an initial perfusion of 20 min with control medium. Mixed disulphides are calculated as *o*-phthalaldehyde-reactive material released from liver extracts after reduction with sodium borohydride minus GSSG and expressed in GSH-equivalents. Data are means ± S.E.M. ($n = 7$ different perfusion experiments).

intensity was measured in an Eppendorf fluorometer at 400–3000 nm (excitation 366 nm) [13, 14]. For calibration 0.1 ml of a standardized GSH solution (3 mM), (calibrated with methylglyoxal and glyoxalase I) was carried along with each determination series instead of the liver powder. The results obtained by the fluorescence method using *o*-phthalaldehyde agreed with those obtained enzymatically with glyoxalase I or with glutathione reductase and 5,5′-dithiobis-(2-nitrobenzoate) [11].

Total glutathione equivalents. Liver powder (50 mg) and 100 mg $NaBH_4$ was weighed into 1.75 ml 0.1 M Tris–HCl, pH 8.0, +2 ml guanidinium chloride (8 M); to prevent foaming, 50 μl n-octanol was added and samples homogenized for 20 sec in an Ultra Turrax [12]. Samples were incubated for 30 min at 40°, then after cooling in an ice bath 1 ml 50% metaphosphoric acid was added to remove excess $NaBH_4$ and to precipitate proteins. After centrifugation for 15 min at 100,000 *g*, the fluorescent adduct with *o*-phthalaldehyde was assayed as described above, and data were expressed on the basis of fluorescence calibration with external GSH.

Mixed disulphides. These were calculated by difference between total glutathione equivalents (GSH plus GSSG), and they were expressed in μmole GSH-equivalents per g wet wt of liver.

Some comments on the nature of the 'mixed disulphides' appear appropriate: mixed disulphides in liver were determined as GSH equivalents released after reduction with a reductant such as borohydride [3–7], and for technical reasons usually a non-enzymatic assay method was applied, making use of the increase in fluorescence intensity of *o*-phthalaldehyde after adduct formation with glutathione [13, 14]. As with other non-enzymatic assay systems applied to biological samples, a disadvantage here was that high specificity was not guaranteed. However, the validity in detecting acid-soluble GSH in liver samples without borohydride treatment was apparent from a comparison of the non-enzymatic method with the two different enzymatic methods mentioned above, all three methods leading to similar values ([11] and further unpublished experiments). This indicates that without the reduction with borohydride the *o*-phthalaldehyde-reactive material in the liver consisted essentially of glutathione. Potentially interfering thiols or related compounds were shown to contribute negligibly to the fluorescence signal [14].

In contrast, the nature of the *o*-phthalaldehyde-reactive material released upon borohydride treatment was not conclusively identified to be GSH, although this appears to be assumed in previous reports [3–7]. We observed only a minor increase in the amount of glutathione in the catalytic assay with DTNB coupled to glutathione reductase. However, the material released by borohydride reacted with *N*-ethyl-maleimide, indicating its thiol nature. In separate experiments (traces not shown), no fluorescence signal with *o*-phthalaldehyde was observed after treatment of borohydride-reduced extracts with *N*-ethyl-maleimide, and also in non-reduced samples.

GSSG. Liver powder (240 mg) was precipitated with 1.2 ml 1 N perchloric acid, 50 mM in *N*-ethylmaleimide, and centrifuged. Of the supernatant 1.1 ml was neutralized to pH 6.2 with 2 M KOH and 0.3 M *N*-morpholinopropane-sulphonic acid. Of the neutralized supernatant 1 ml was applied to a QAE Sephadex A 25 column and GSSG eluted as described in [11]. GSSG was measured by following oxidation of NADPH at 340–400 nm in a Sigma ZWS II dual-wavelength spectrophotometer (Biochem Co., München-Puchheim, West Germany). Intracellular GSSG contents are expressed in nmole per g wet wt of liver after correcting for biliary GSSG contents using a bile space of 2.3 μl per g wet wt liver [15].

NADPH and NADP⁺. These were assayed according to Ref. [16].

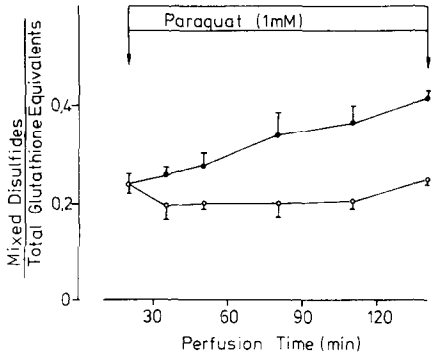

Fig. 2. Mixed disulphide content in relation to total glutathione equivalents during perfusion of rat livers. Control (○), and paraquat, 1 mM (●) after 20 min perfusion with control medium.

RESULTS

Influence of paraquat on the glutathione status. During the perfusion of rat liver the content of total glutathione in the cell decreased from 7.5 to

Table 1. Glutathione status in haemoglobin-free perfused rat liver under the influence of paraquat

	Total glutathione	GSH	Mixed disulphides	GSSG
	(μmole/g liver wet wt)			(nmole/g)
Control, 20 min perfusion	7.54 ± 0.26	5.90 ± 0.24	1.80 ± 0.11	18.3 ± 0.5
140 min perfusion	6.20 ± 0.17	4.64 ± 0.19	1.71 ± 0.23	17.0 ± 3.7
Paraquat (1 mM), 140 min perfusion	5.96 ± 0.13	3.54 ± 0.17	2.54 ± 0.08	38.1 ± 1.7

Livers were perfused for 140 min. When paraquat was added, the perfusate contained paraquat from 20 to 140 min. Samples were taken by freeze-stop and analysed as described in Materials and Methods. Data are given as means ± S.E.M. (n = 8 different perfusions).

6.2 μmole/gram liver wet weight over the period between 20 and 140 min of perfusion (Table 1). This decrease was exclusively due to a release of glutathione from the hepatocytes, occurring at a rate of 10–12 nmole/min per gram wet weight as described previously [17]. The cellular GSH content decreased by 1.3 μmole/gram liver wet weight, whereas the levels of mixed disulphides and of GSSG remained essentially unchanged.

In the presence of paraquat, infused at a constant concentration of 1 mM over the period from 20 to 140 min, the decrease in total glutathione was 1.6 μmole/gram liver wet weight, i.e. slightly higher than in the controls. However, the loss of GSH was 2.4 μmole/gram liver wet weight, 1.1 μmol higher than in the controls. A substantial part of this difference was accounted for by an increase in the mixed disulfides by 0.7–0.8 μmole/gram liver wet weight as compared to the controls. The remainder of the difference was accounted for by the increased biliary release of GSSG and GSH, amounting to 0.4 μmole GSH equivalents as described previously [2].

Thus, paraquat induced a marked rearrangement in the amount of intracellular glutathione equivalents present as GSH or as mixed disulphide. The time-course of this rearrangement shows that stable changes appeared at a time of about 30 min after onset of the paraquat infusion (Fig. 1). The ratio of the mixed disulphides to the total glutathione equivalents was about 0.2 in the controls and rose to about 0.4 with paraquat (Fig. 2).

The mixed disulphides were practically totally protein-bound so that low molecular weight mixed disulphides were quantitatively negligible. When 100 mg liver powder were first precipitated with 0.5 ml 1 N perchloric acid, and the washed pellet was analysed for mixed disulphides the values were similar to the results from total liver powder, 102 ± 4% (n = 10). With these protein pellets no free glutathione was detectable, so that all thiol released upon borohydride treatment originated from protein-bound mixed disulphides.

Glutathione redox state. Intracellular glutathione disulphide levels, corrected for GSSG in the biliary space, increased from 18 to 38 nmole GSSG/g liver wet wt in the presence of paraquat (Table 1), lowering the ratio of GSH/GSSG from 273 to 93 at 140 min of perfusion. The time-course of the level of intracellular GSSG is shown in Fig. 3.

As stated above, it appears possible that an increased concentration of intracellular GSSG gave rise to the formation of mixed disulphides via reaction (1), catalysed by thioltransferase(s). The relationship between mixed disulphide levels and GSSG is shown in Fig. 4.

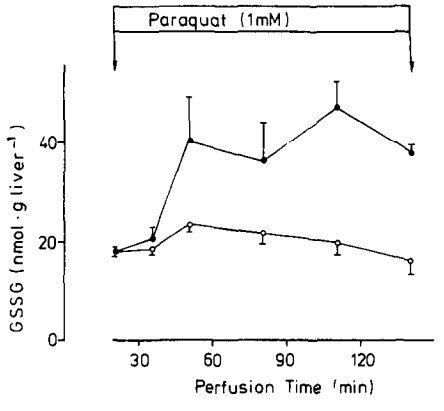

Fig. 3. Influence of paraquat on intracellular levels of GSSG in perfused rat liver. GSSG was estimated after trapping of GSH with *N*-ethyl-maleimide and values corrected for GSSG secreted into the bile (see Materials and Methods). Control (○), and paraquat (●). Data are means ± S.E.M. (n = 4 different perfusion experiments).

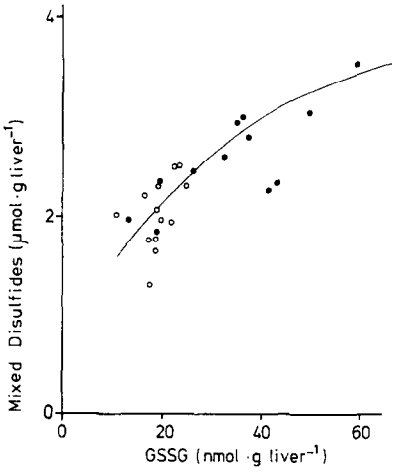

Fig. 4. Relationship between intracellular mixed disulphides and GSSG levels in rat liver. Different points are values obtained from aliquots of livers perfused for different times with (●) and without (○) paraquat (1 mM).

NADP(H) contents. The hepatic contents of NADPH and $NADP^+$ were also measured. The ratio of contents, NADPH/$NADP^+$, at 140 min of perfusion time was 5.1 ± 0.1 ($n = 5$) in the controls, and it decreased to 2.3 ± 0.3 ($n = 6$) when paraquat was present, starting at 20 min perfusion time. The sum of the cellular amounts, NADPH + $NADP^+$, were unchanged, amounting to 315 ± 23 and 339 ± 35 nmole/g wet wt in controls and paraquat perfusions, respectively.

DISCUSSION

Perturbation of glutathione status by paraquat. The results clearly demonstrate that paraquat causes significant changes in the intracellular glutathione system of liver. The changes concern the disposition between free and bound GSH as well as the redox state. The increase in mixed disulphides represents the loss of glutathione that was unaccounted for in Ref. [2].

An increase in mixed disulphides may result in various consequences for cellular metabolism. For example, activities of enzymes are regulated by formation of mixed disulphides, as has been reported for fructose diphosphatase [18], pyruvate kinase [19, 20], glycogen synthetase [21], and phosphorylase phosphatase [22]. The effect of disulphides on liver fructose diphosphatase is an activation whereas with the latter three enzymes it is an inhibition. Thus, an increase in the content of GSSG may stimulate formation of mixed disulphides, thereby activating processes leading to higher levels of glucose-6-phosphate. Glucose-6-phosphate is needed for the production of NADPH in the glucose-6-phosphate dehydrogenase reaction necessary for the reduction of GSSG. In addition, further sites of cellular thiol groups may be transformed into mixed disulphides, possibly leading to cytotoxic effects yet to be characterized.

Possible mechanisms. The mechanism by which paraquat affects GSSG concentrations can involve several factors. First, there may be a relation to the proposed mechanism of toxicity, lipid peroxidation [23]. In cells paraquat undergoes a cyclic reduction and oxidation with the concomitant production of superoxide anions ($O_2^{\cdot-}$) which may, in turn, produce more active oxygen species such as singlet oxygen and $OH^{\cdot}$-radicals capable of initiating lipid peroxidation [24]. Recently, it was shown that $^{\cdot}OH$ can be produced by a direct reaction of the paraquat radical with H_2O_2 [25], and also the formation of a crypto-$^{\cdot}OH$ species has been proposed [26]. In perfused liver, paraquat was shown to lead to ethane production when no substrates were present [27] (as is the case also in this work), but in the presence of lactate and pyruvate [27] or in the intact animal [28] no extra ethane production was observed except in selenium-deficiency [29]. While there are doubts regarding lipid peroxidation as mechanism of paraquat toxicity [30], an involvement of radical reactions in the present experiments therefore remains a possibility.

A second explanation for the stimulated oxidation of GSH may be the consumption of NADPH by paraquat via redox cycling. It is known that paraquat stimulates the pentose-phosphate pathway in rat lung [31, 32], and it decreases the content of NADPH by about 50% in lung [33] and in liver (see Results). If the rate of paraquat reduction becomes excessive, the concentration of NADPH will fall below that required to sustain physiological processes. This was shown for fatty acid synthesis which was inhibited in paraquat-treated lungs [34]. Only a small fraction of 3% of the GSSG produced in the GSH-peroxidase system, or independent from it, is released from the liver [35]. Because most of the GSSG is reduced back by the GSSG-reductase with NADPH as reductant, GSSG will be released or may be transferred to mixed disulphides by reaction (1) when NADPH supply becomes rate-limiting.

A possibility to be discussed is the formation of a paraquat–glutathione conjugate. In the present experiments, there is no difference in total glutathione equivalents between paraquat addition and control (Fig. 1, Table 1), so that there is no additional intracellular glutathione to be accounted for. Furthermore, there is no indication ([34, 36]; and unpublished work, R.B.) on the existence of such a conjugate.

Relationship between GSSG and mixed disulphide levels. The amount of mixed disulphides, 1.8 μmole/g wet wt (Table 1), is similar to that found in previous reports [5, 7] and, with a protein content of 191 ± 4 mg/g wet wt ($n = 12$), is 9.4 nmole/mg cellular protein, similar to the 10.4 nmole/mg protein reported in [6]. Although the equilibrium constant for reaction (1) is near unity, the ratio of the contents of mixed disulphides to GSSG is 80–100 (Fig. 4). This is thought to be due to the nonhomogeneous phase system that exists for the thioltransferase, with bulk phase GSSG on the one hand and with the largely protein-bound and membrane-bound mixed disulphides on the other. Thus, it is entirely possible that thioltransferase activities are sufficiently high to catalyse localized equilibria.

A double-reciprocal plot of the data in Fig. 4 shows that a saturation of potential cellular mixed disulphide sites may be reached at a value of near 4 μmole/g liver. Occupancy of potential mixed disulphide sites thus is about 1/2 in the controls and is augmented to about 2/3 in the presence of paraquat. Identification of the particular sites of protein mixed disulphide formation will be an important next step.

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