FORMATION AND EFFLUX OF GLUTATHIONE DISULFIDE STUDIED IN ISOLATED RAT HEPATOCYTES

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1. Introduction

The efflux of intracellular GSH and GSSG to the extracellular space has been studied in various experimental systems including the isolated, perfused liver [1,2] and suspensions of isolated hepatocytes [3,4] under various metabolic conditions. It appears that both GSH and GSSG are continuously released from liver cells into plasma and bile, respectively, and that their translocation may be mediated by distinct transport systems, since neither GSH nor GSSG readily diffuses through the plasma membrane. Release of GSH from the liver may be the major source of plasma GSH and GSSG [5,6], and GSSG efflux into bile may reflect the operation of a transport system normally involved in the secretion of glutathione conjugates [5].

The release of GSSG from the isolated, perfused liver, or from isolated hepatocytes, is markedly increased by exogenously added organic hydroperoxides [1,7], as well as under conditions of enhanced rates of endogenous H_2O_2 generation [8]. This phenomenon is therefore thought to reflect the intracelluar activity of glutathione peroxidase, a cytosolic selenoprotein that catalyzes the reduction of H_2O_2 and organic hydroperoxides with the concomitant oxidation of GSH to GSSG. However, only a fraction of the GSSG that is formed in the glutathione peroxidase reaction is actually released from the hepatocytes [4], and most of the GSSG is reduced back to GSH by the flavoprotein, glutathione reductase, which utilizes NADPH as a reductant.

Abbreviations: GSH, glutathione, reduced form; GSSG, glutathione disulfide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; CDNB, 1-chloro-2,4-dinitrobenzene

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Here, we extend our investigation of formation and secretion of GSSG by isolated hepatocytes [4]. Stimulation of GSH oxidation by glutathione peroxidase has been achieved by incubation of hepatocytes with agents that induce enhanced intracellular H_2O_2 formation, or by addition of low concentrations of H_2O_2 to the medium. Intra- and extracellular concentrations of GSH and GSSG have been measured and the results compared to changes in the intracellular NADPH/$NADP^+$ redox level. The purpose of this research has been to provide additional information about the processes that control the formation and efflux of intracellular GSSG.

2. Materials and methods

Collagenase (grade II) was obtained from Boehringer (Mannheim) and GSH and GSSG were from Sigma Chemicals (St Louis MO). Other chemicals were at least of reagent grade and purchased from local commercial sources.

Hepatocytes were isolated from fed, male Sprague-Dawley rats (200–250 g) as in [9]. Rats received sodium phenobarbital in the drinking water (1 mg/ml) for 5 days before use. Isolated hepatocytes were quantitated, and their viability determined, by counting the cells in a Buerker chamber in presence of 0.2% trypan blue.

Incubations were performed in rotating, round-bottom flasks (10^6 cells/ml) at 37°C in a modified Krebs-Henseleit buffer (pH 7.4) supplemented with 25 mM Hepes. Incubations were gassed continuously with carbogen (95% O_2, 5% CO_2). Viability during the course of experiments was determined by the NADH penetration assay [10].

GSH was measured by the colorimetric assay in

[11] and GSH and GSSG by the fluorometric method in [12]. The validity of these assays has been established under similar conditions by use of high-performance liquid chromatography [13]. $NADP^+$ and NADPH concentrations were assayed by the spectrophotometric method in [14].

Samples for measuring the glutathione conjugate of CDNB were taken from cell incubations, centrifuged and the light absorption at 340 nm of the supernatant was determined in an Aminco DW-2 spectrophotometer.

3. Results

When freshly isolated hepatocytes were incubated in modified Krebs-Henseleit buffer (pH 7.4) there was a slow, continuous decrease in cellular GSH level which corresponded to a similar increase in GSSG concentration in the medium (fig.1(A)). In agreement with [3], this process was accelerated when ethylmorphine (1 mM) was included in the medium. As shown in fig.1(B) incubation with this drug was also associated with a moderate increase in intracellular GSSG concentration. The latter phenomenon was much more pronounced when ethylmorphine was replaced by menadione to stimulate intracellular H_2O_2 production; menadione is reduced to the corresponding semiquinone by cellular flavoproteins, primarily NADPH-cytochrome *P*-450 reductase, and then enters a redox cycle with molecular oxygen which results in generation of H_2O_2 [15]. As shown in fig.1(C,D) incubation of hepatocytes with increasing concentrations of menadione resulted in rapid increase in intracellular GSSG concentration which then declined slowly during continuing incubation.

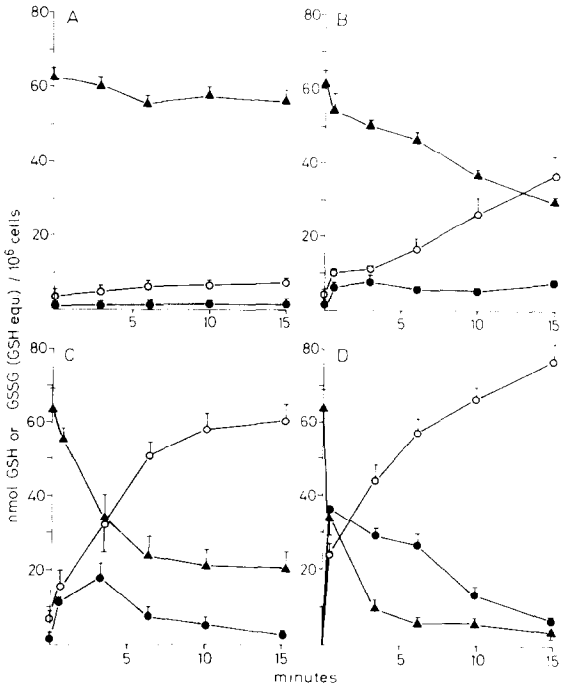

Fig.1. GSH and GSSG levels in hepatocytes incubated in the absence and presence of ethylmorphine and menadione: (A) control; (B) 1 mM ethylmorphine; (C) 0.05 mM menadione; (D) 0.2 mM menadione; (▲——▲) intracellular GSH; (●——●) intracellular GSSG; (○——○) extracellular GSSG. Mean and standard error of 3 expt.

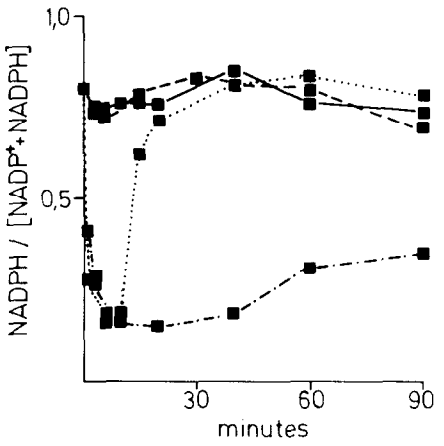

Fig.2. Effect of ethylmorphine and menadione metabolism on the [NADPH]/[$NADP^+$ + NADPH] ratio in isolated hepatocytes: (■——■) control; (■– – –■) 1 mM ethylmorphine; (■. . .■) 0.05 mM menadione; (■.–.–.■) 0.2 mM menadione. One experiment typical of 3.

Incubation of hepatocytes with menadione (0.05 mM or 0.2 mM) was also associated with a rapid and marked change in the cellular NADPH/$NADP^+$ ratio (fig.2), and it appears quite possible that this altered redox level may have caused the initial increase in intracellular GSSG level observed under these conditions. With ethylmorphine however, no measurable change in cellular NADPH/$NADP^+$ ratio was observed, and yet, incubation of hepatocytes with this drug also led to loss of cellular GSH due to GSSG secretion.

Stimulation of endogenous H_2O_2 production by ethylmorphine or menadione involves the microsomal cytochrome *P*-450 system and thus an enhanced consumption of cytosolic NADPH via this pathway. Sub-

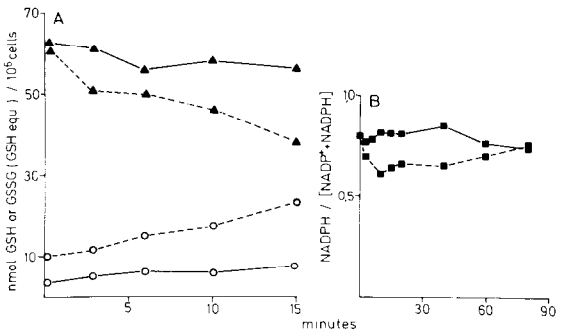

Fig.3. Effect of H_2O_2 metabolism on the levels of GSH and GSSG and on the [NADPH]/[$NADP^+$ + NADPH] ratio in isolated hepatocytes. (A) Levels of: (▲) intracellular GSH; (○) extracellular GSSG; in the absence (——) and presence (– – –) of H_2O_2 (10 nmol added to 20 ml incubations/min). (B) (■) Ratio of [NADPH]/[$NADP^+$ + NADPH] in the absence (——) and presence (– – –) of H_2O_2 (10 nmol added/min). One experiment typical of 3.

sequent metabolism of the hydrogen peroxide by glutathione peroxidase further increases the utilization of cytosolic NADPH. To avoid this dual effect on the cytosolic NADPH pool, a series of experiments was performed where glutathione peroxidase was activated by exogenously added H_2O_2. The concentration of H_2O_2 was kept constant in the medium at a level which has previously been found to correspond to the maximal capacity of isolated hepatocytes to metabolize H_2O_2 [4]. As shown in fig.3, incubation of hepatocytes under these conditions resulted in loss of cellular GSH and a corresponding increase in the GSSG content in the medium. Intracellular accumulation of GSSG was not observable, and there was only a minor effect on the cellular NADPH/$NADP^+$ ratio.

Finally, a series of experiments was performed in an attempt to explore the possibility that a common mechanism may be involved in the release of GSSG and glutathione conjugates by liver cells. Hepatocytes were incubated in the absence or presence of CDNB and H_2O_2 in the medium. However, the results of these experiments were negative since there was no observable effect on the H_2O_2-stimulated rate of release of GSSG by hepatocytes in the presence of CDNB (fig.4(A)). Neither was the rate of secretion of the glutathione conjugate of CDNB affected under conditions of enhanced GSSG secretion by the cells (fig.4(B)). The results therefore suggest that different transport mechanisms may be involved in the release by hepatocytes of GSSG and a glutathione conjugate, or that the release is mediated by a common mechanism which was non-saturable under our experimental conditions.

4. Discussion

Incubation of isolated rat hepatocytes in a buffered salts solution is associated with a slow, steady decrease in intracellular GSH level [16]. This is abolished when glutathione precursor amino acids are included in the medium. Under both conditions there appears to be a release of GSH as well as GSSG from the cells into the medium, and the intracellular concentration of GSSG is normally kept very low. It is probable that glutathione reductase activity is of major importance for the maintenance of a high GSH/GSSG ratio in the cells.

NADPH is used as the reductant in the glutathione reductase reaction and a lowered NADPH/$NADP^+$ ratio has been suggested to be responsible for the release of intracellular GSSG observed under conditions of enhanced glutathione peroxidase activity [1]. It now appears, however, that an enhanced rate of GSSG release by the cells can be seen also when there are no, or only minor changes in the cellular NADPH/$NADP^+$ ratio. This was true during incubation of

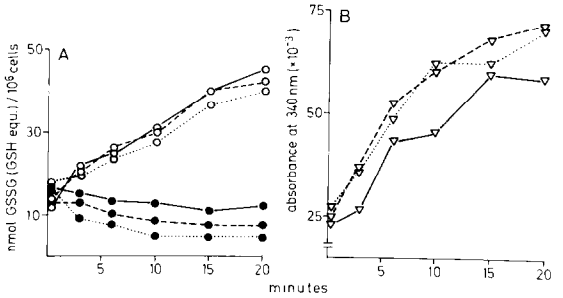

Fig.4. Combined effects of CDNB and H_2O_2 on the release of GSSG and GSH conjugates from isolated hepatocytes. (A) Effect of CDNB on the release of GSSG from isolated hepatocytes in the presence of H_2O_2. H_2O_2 was added to the incubations (20 ml) at 10 nmol/min: (○) extracellular GSSG concentration; (●) intracellular GSSG concentration; (——) without CDNB; (– – –) 0.025 mM CDNB; (. . .) 0.05 mM CDNB. (B) Effect of H_2O_2 on GSH–CDNB conjugate release from isolated hepatocytes, in the presence of 0.05 mM CDNB: (▽——▽) without H_2O_2; (▽– – –▽) 10 nmol H_2O_2 added to 20 ml incubations/min; (▽. . .▽) 20 nmol H_2O_2 added to 20 ml incubations/min. One experiment typical of 3.

hepatocytes with either ethylmorphine or exogenously added H_2O_2. However, the results of the experiments with menadione suggest that intracellular accumulation of GSSG may be directly related to the decreased NADPH/$NADP^+$ ratio.

It therefore appears that lack of NADPH is not the only reason for the release of GSSG by hepatocytes. Stimulation of glutathione peroxidase activity can result in enhanced rate of GSSG secretion by the cells also in the absence of any measurable change in the cellular NADPH/$NADP^+$ ratio. This suggests that the capability of the cells to reduce formed GSSG back to GSH is influenced by other factors in addition to the availability of NADPH. The relationship between overall intracellular glutathione reductase activity and GSSG release from isolated hepatocytes is under investigation.

The fact that extracellular GSSG is not taken up by isolated hepatocytes, and the evidence for selective secretion of GSSG via the bile in the isolated, perfused liver [17] suggest that the release of GSSG from liver cells is mediated by an active mechanism and does not occur by simple diffusion. The possibility that a common transport system may be involved in the release of both GSSG and glutathione conjugates has been suggested [4], but has not received experimental support here. However, this evidence is not conclusive, and further experimentation is required to characterize hepatic GSSG secretion in detail to prove or disprove the existence of a common transport system for GSSG and glutathione conjugates.

Acknowledgement

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References

[1] Oshino, N. and Chance, B. (1977) Biochem. J. 162, 509–525.
[2] Bartoli, G. and Sies, H. (1978) FEBS Lett. 86, 89–91.
[3] Jones, D. P., Thor, H., Andersson, B. and Orrenius, S. (1978) J. Biol. Chem. 253, 6031–6037.
[4] Jones, D. P., Eklöw, L., Thor, H. and Orrenius, S. (1981) submitted.
[5] Sies, H., Wahlländer, A. and Whaydhas, C. H. (1978) in: Functions of Glutathione in Liver and Kidney (Sies, H. and Wendel, A. eds) pp. 120–125, Springer-Verlag, Berlin, Heidelberg.
[6] Griffith, H. and Meister, A. (1979) Proc. Natl. Acad. Sci. USA 76, 5606–5610.
[7] Sies, H., Gerstenecker, C., Henzel, H. and Flohé, L. (1972) FEBS Lett. 27, 171–175.
[8] Chance, B., Sies, H. and Boveris, A. (1979) Physiol. Rev. 59, 527–605.
[9] Moldéus, P., Högberg, J. and Orrenius, S. (1978) Methods Enzymol. 51, 60–71.
[10] Högberg, J. and Kristoferson, A. (1977) Eur. J. Biochem. 74, 77–82.
[11] Saville, B. (1958) Analyst 83, 670–672.
[12] Hissin, P. J. and Hilf, R. (1976) Anal. Biochem. 74, 214–226.
[13] Jones, D. P., Moldéus, P., Stead, A. H., Ormstad, K., Jörnvall, H. and Orrenius, S. (1979) J. Biol. Chem. 254, 2787–2792.
[14] Klingenberg, M. (1970) in: Methoden der Enzymatischen Analyze (Bergmeyer, H. U. ed) pp. 1975, Verlag Chemie, Weinheim.
[15] McCord, J. M. and Fridovich, I. (1969) J. Biol. Chem. 245, 1374–1377.
[16] Thor, H., Moldéus, P. and Orrenius, S. (1979) Arch. Biochem. Biophys. 192, 405–413.
[17] Bartoli, G. H., Häberle, D. and Sies, H. (1978) in: Functions of Glutathione in Liver and Kidney (Sies, H. and Wendel, A. eds) pp. 27–31, Springer-Verlag, Berlin, Heidelberg.