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SHORT COMMUNICATIONS

Effect of hypoxic perfusion on hepatic concentration and biliary release of glutathione disulfide

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One of the mechanisms by which hepatocytes maintain a very low GSSG to GSH ratio is the transport of GSSG* into the bile. Akerboom *et al.* [1] showed that the release of GSSG in the bile correlates well with hepatic GSSG concentration under conditions of oxidative stress which cause increased GSSG formation. Because of these findings, biliary GSSG release has been used as an index of oxidative stress in the liver.

Reactions which result in the formation of GSSG are generally considered to be oxygen dependent. Hypoxic conditions might be expected to diminish GSSG formation and lead to a fall in biliary GSSG release. If this occurs, hepatic oxygen tension will have to be taken into consideration when biliary GSSG release is used as an index of oxidative stress. The experiments presented here were carried out to determine how a decrease in delivery of oxygen to the liver would affect hepatic and biliary GSSG and GSH.

Materials and methods

Livers were obtained from 200 to 400 g male Sprague–Dawley rats purchased from Harlan Sprague–Dawley, Indianapolis, IN. The rats were fed Teklad pelleted rat diet *ad lib.* and anesthetized prior to experimentation with sodium pentobarbital (50 mg/kg) administered intraperitoneally.

Liver perfusions were carried out as described previously [2]. The bile duct was cannulated with PE-50 tubing and bile was collected during sequential 5-min intervals into preweighed tubes containing 0.25 ml of 3% meta-phosphoric acid. Then the tubes were reweighed to determine bile flow. Total glutathione and GSSG in bile were measured by the method used previously [2]. Krebs–Henseleit buffer was warmed to 37° and perfused at 3–4 ml/g·min. The apparatus employed had two buffer-delivery systems, each consisting of a pump, a warming coil, and an oxygenator. The two systems were attached to a valve, allowing rapid switching from one to the other. Adjacent to the valve was a Clark-type oxygen electrode providing constant monitoring of the buffer oxygen tension. The oxygen electrode was calibrated to zero with sodium hydrosulfite. The gas flowing through one oxygenator was 95% O_2:5% CO_2 and the gas flowing through the other was 95% N_2:5% CO_2.

The experimental protocol (Fig. 1) consisted of a 20-min stabilization, a 15-min hypoxic perfusion, and a 25-min recovery period. The perfusion was carried out with the oxygenated buffer except during the hypoxic perfusion period when the buffer equilibrated with 95% N_2:5% CO_2 was perfused. The flow rate was measured and caval perfusate was sampled every 5 min for glutathione and lactic dehydrogenase determinations [2].

Some livers were freeze-clamped with large aluminium tongs at liquid N_2 temperature at 35 min of the protocol. The crushed liver was ground to a powder using a mortar and pestle at the same temperature. Liver powder (0.5 g) was added to a tube containing 3 ml of 10% trichloroacetic acid. Total glutathione was measured using the recirculating assay as described before [3]. For GSSG measurement the sample was treated with 80 μmol of NEM to derivatize the GSH, and the pH was adjusted to 6.0–6.2 using 2 M KOH, 0.3 M MOPS. After centrifugation, the supernatant fraction was extracted three times with 4 vol. of diethyl ether to remove excess NEM. A 0.3-ml aliquot of the aqueous solution was applied to a C18 Sep-Pak column, and the GSSG was eluted with 1 ml of 0.125 M sodium phosphate, pH 7.5. The Sep-Pak treatment was repeated with 0.5 ml of the eluate from the first column, and the resulting eluate was assayed for GSSG using the recirculating method [3]. The recovery of the GSSG added to samples was 103 ± 14%, N = 17. GSH was calculated as the difference between total glutathione and GSSG.

GSH, GSSG, and glutathione reductase (yeast type II) were obtained from the Sigma Chemical Co., St. Louis, MO. NADPH was purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. All other chemicals used were of reagent grade or better.

Results

The effects of a brief period of hypoxia on the perfused liver are shown in Fig. 1. The hypoxic period was limited to 15 min to avoid irreversible liver injury, as indicated by lactic dehydrogenase release. Release of glutathione into the caval perfusate, which is virtually all in the form of GSH, was unaffected by the hypoxic period (Fig. 1A). Bile flow and release of GSH into bile were also unaffected as shown in Fig. 1B.

Hypoxic perfusion had a striking effect on biliary GSSG release (Fig. 1C). GSSG release fell with hypoxia to less than 10% of the oxygenated value and returned to initial levels after oxygen was readmitted to the system. The 5-min lag between the start of the hypoxic period and the fall in biliary GSSG release was related to the volume of the bile in the biliary cannula. This result contrasted with perfusions in which oxygen supply was maintained and in which no decrease in biliary GSSG release occurred during the 60-min perfusion [2].

In an attempt to correlate intracellular GSSG with biliary GSSG release, perfused livers were freeze-clamped at 35 min and assayed for GSSG and total glutathione. Perfusions in which the buffer was oxygenated throughout and perfusions which incorporated a hypoxic period from 20 to 35 min were used. Table 1 shows the liver GSH and GSSG concentrations. Hypoxic perfusion had no significant effect on GSH concentration. However, liver GSSG concentration fell to 63% of the oxygenated value during hypoxic perfusion. Thus, there was a difference between the effect of hypoxia on GSH and its effect on GSSG.

Discussion

Hepatic GSSG concentration in oxygenated livers was 60 ± 21 nmol GSH equivalents/g after 35 min of perfusion. This concentration is somewhat higher than the value of 36 nmol GSH equivalents/g reported by Akerboom *et al.* after 42 min of perfusion [1], but considerably less than that of most other reports. Higher values probably result from

* Abbreviations: GSSG, glutathione disulfide; GSH, reduced glutathione; NEM, *N*-ethylmaleimide; MOPS, 3(*N*-morpholino) propanesulfonic acid; and LDH, lactic dehydrogenase.

Table 1. Effect of hypoxic perfusion on hepatic GSH and GSSG*

	N	GSH (nmol GSH equivalents/g)	GSSG (nmol GSH equivalents/g)
Oxygenated perfusion	8	4170 ± 760	60 ± 21
Hypoxic perfusion	9	4780 ± 1430	38 ± 13†

* Livers were freeze-clamped after 35 min of perfusion. Hypoxic perfusion was carried out as in Fig. 1. Values are means ± SD.

† Significantly different from oxygenated perfusion ($P < 0.05$) by Student's unpaired *t*-test.

oxidation of GSH if the tissue is not freeze-clamped or from GSH not quantitatively removed before measuring GSSG. In the present study the tissue was collected by freeze-clamping and conjugation of GSH with NEM was used to minimize interference by GSH in the estimation of GSSG. The brief hypoxic period used in this study affected only GSSG since neither liver GSH nor biliary GSH release changed significantly during the hypoxic period. Longer exposure to hypoxia, however, may decrease liver GSH by limiting the availability of ATP for its synthesis.

Known mechanisms for GSSG formation are oxygen-dependent, and it is not surprising that perfusion with oxygen-free buffer decreased tissue and biliary GSSG. However, to the best of our knowledge, the work described in this report is the first direct demonstration that maintenance of GSSG concentration in liver requires oxygen. Decreased GSSG in ischemic kidneys tissue has been reported [4], but Wendell [5] failed to detect a significant change in GSSG in rat hearts perfused with anaerobic buffer. The reasons for the latter finding are not clear since preliminary studies in this laboratory suggest that GSSG concentration also decreases in hypoxic perfused rat hearts. Evidence has been presented that sources of GSSG formation other than glutathione peroxidase exist [6].

Biliary GSSG release was more sensitive to oxygen supply than was tissue GSSG concentration. There are several potential explanations for this. One is that release of GSSG may vary in different zones of the hepatic lobule and hypoxic perfusion may affect the zones in different ways. Another is that GSSG may be distributed non-uniformly in the hepatocyte with a relatively low concentration near the biliary canaliculus. Further studies will be necessary to evaluate these possibilities.

These results indicate that oxygen supply to the liver will have to be considered when biliary GSSG release is used as an indicator of oxidative stress. Figure 1 shows that increasing perfusate oxygen can cause a several-fold rise in biliary GSSG release. Thus, an increase in biliary GSSG release could result from improved oxygenation of the liver and is not a specific indicator of oxidative stress.

In summary, changes in the biliary efflux of GSSG as a function of intracellular GSSG concentration were investigated in rat livers perfused with oxygenated and oxygen-depleted buffers. Biliary efflux of GSSG decreased 91% and tissue GSSG decreased 37% during hypoxia. However, hypoxic perfusion had no effect on biliary GSH release, liver GSH content, or bile flow. These results demonstrate that liver and bile GSSG are dependent on oxygen supply to the liver.

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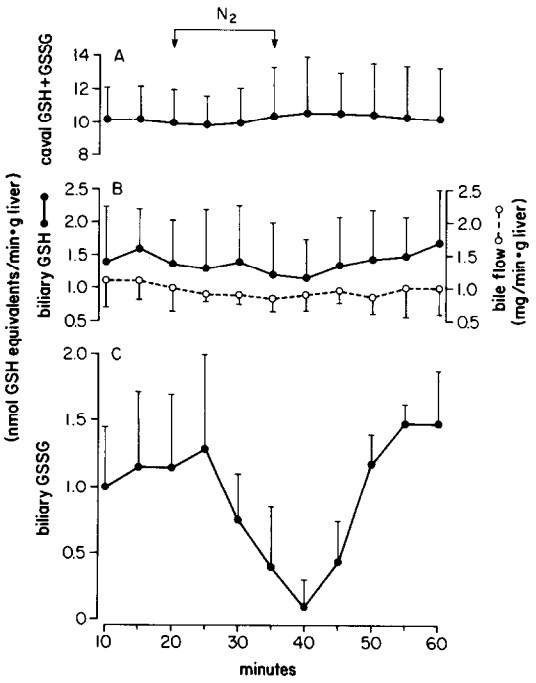

Fig. 1. Effect of hypoxia on GSSG release by perfused rat liver. Livers were perfused as described in Materials and Methods with a hypoxic period from 20 to 35 min. Oxygen tension in the buffer entering the liver during the hypoxic period was less than 5% that during the oxygenated period as determined by the oxygen electrode. Lactic dehydrogenase release did not rise during or after hypoxia. Values are means of five to nine livers. The bracket represents one SD.

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Ketamine protects acetylcholinesterase against *in vitro* inhibition by sarin

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Ketamine (2-(*o*-chlorophenyl)-2-methylamino-cyclohexone) is a drug that can be used for anesthetic, analgetic and sedative purposes. Several mechanisms have been proposed for this versatile substance [1]. As anesthetics in general, ketamine induces changes in the membrane fluidity, e.g. in synaptosomes and mitochondria [2]. It also has ion channel blocking properties [3]. Furthermore, several central nervous transmitter systems, including the cholinergic one, are affected during ketamine anesthesia [1].

Our interest in this drug and its interaction with the cholinergic system was raised during a study on pigs, intoxicated by an anticholinesterase compound [4]. Animals were anesthetized either by ketamine or by sodium pentobarbital. We observed that the ketamine-treated pigs tolerated more of the anticholinesterase (soman) than did pigs pretreated with pentobarbital. This difference in sensitivity towards the cholinesterase inhibitor could mean that pentobarbital potentiates the toxicity or that ketamine reduces the toxicity or both. The first hypothesis finds some support from a work by Clement [5], who claims that pentobarbital enhances the toxic effect of soman in mice. The second hypothesis is supported by recent findings by Klemm [3]. He observed that ketamine protects against intoxication by organophosphates, and he suggested that this protection is due to the ionic channel blocking properties.

It is, however, possible that such a protection could at least partly be mediated by protection of the target enzyme acetylcholinesterase (EC 3.1.1.7), as it has been shown that ketamine is a reversible inhibitor of this enzyme, both *in vitro* and *in vivo* [6].

The interactions between ketamine, acetylcholinesterase from bovine brain and the potent organophosphorous inhibitor sarin (isopropyl methylphosphonofluoridate) have now been studied. The study also includes results on the influence of ketamine on reactivation and dealkylation ("aging") of sarin-inhibited enzyme, i.e. reactions of importance for the therapeutic countermeasures against organophosphate intoxication.

Materials and methods

Ketamine hydrochloride was a gift from Parke Davis Co. and HI-6 dichloride from Dr J. Clement, DRES, Canada. Sarin was synthesized at the Chemistry Department of this institute.

Acetylcholinesterase was purified from bovine brain with affinity chromatography, as described recently [7]. The preparation had a specific activity of 1.5 μkat mg^{-1}. Enzyme activity was measured by Ellman's procedure [8], using 1 mM acetylthiocholine as substrate.

Inhibition by ketamine. The reversible inhibition constants were determined, using ketamine in the concentration range 0.5–2 mM and varying the substrate concentration between 0.1 and 0.9 mM. These experiments were performed at 22°, in 0.1 M sodium phosphate buffer, 0.1% Triton X-100, pH 8.0. Calculations were based on Eadie–Hofstee plots.

Sarin inhibition. Enzyme, sarin and ketamine were incubated in 0.1 M sodium phosphate buffer, 0.1% Triton X-100, pH 7.4 at 22°. Sarin concentration was varied between 0.05 and 0.5 μM and ketamine concentration between 0.5 and 5 mM; no ketamine in control experiments. Aliquots from the incubation media were taken in a time series, usually every second minute. The irreversible inhibition constant, k_i, was determined as the rate constant, obtained by linear regression, divided by the sarin concentration used.

Aging rate constant, k_a, *and reactivation efficacy.* Enzyme was inhibited by 0.5 μM sarin for 5 min at pH 7.4. Excess sarin was removed by gel filtration on a Pharmacia PD-10 column (Sephadex G-25 gel), equilibrated with 0.067 M sodium phosphate buffer, 0.1% Triton X-100, pH 6.9. Uninhibited enzyme was treated in the same way. The enzyme solutions thus obtained, denoted EI and E respectively, were used for the aging and reactivation experiments.

For the aging rate experiments, two 0.4 ml samples of E and EI, respectively, were transferred to tubes containing 1.6 ml 0.067 M sodium phosphate buffer, with 0.1% Triton X-100, pH 6.9, with and without 5 mM ketamine, at 37°. The experimental conditions (ionic strength, pH and temperature) were chosen to give a rather rapid aging. From these tubes 2 × 0.1 ml aliquots were withdrawn in a time series, t = 0–5 hr, and transferred to tubes containing 0.3 ml 0.1 M sodium phosphate buffer, 0.1% Triton X-100, pH 7.4, with and without 0.075 mM HI-6. Enzymatic activity was measured after 30 min of reactivation. Percentage reactivation and the rate constant of aging were calculated according to Keijer and coworkers [9].

Reactivation experiments. These experiments were performed at pH 7.4, 0.1 M sodium phosphate buffer, 0.1% Triton X-100, obtained by diluting E and EI 20 times. The oxime (HI-6) concentration was varied between 0.01 and 0.075 mM. The series with ketamine were done at a concentration of 5 mM. Proper controls such as activity of E and EI in the absence of oximes were also run. Aliquots were withdrawn in time series, usually t = 5–400 min, the activity measured and percentage reactivation calculated as described above. The rate constant, k_{obs}, was calculated from Guggenheim plots, ln (% react$_{t=\infty}$ − % react$_t$) vs t, where reactivation at t$_\infty$ was estimated as the value obtained after 24 hr. The bimolecular reaction rate constant, k_2/K_d, which reflects reactivation efficacy, was obtained as the slope of a plot $1/k_{obs}$ versus 1/(HI-6).

Results and discussion

We could confirm previously reported results that ketamine inhibits acetylcholinesterase, with a mixed type of