Free Rad. Res. Comms., Vols. 12-13, pp. 737-743
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VASCULAR OXIDANT STRESS AND HEPATIC ISCHEMIA/REPERFUSION INJURY

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(Received September 17, 1989)

The objective of this study was to test the hypothesis that the extracellular oxidation of glutathione (GSH) may represent an important mechanism to limit hepatic ischemia/reperfusion injury in male Fischer rats *in vivo*. Basal plasma levels of glutatione disulfide (GSSG: 1.5 ± 0.2 μM GSH-equivalents), glutathione (GSH: 6.2 ± 0.4 μM) and alanine aminotransferase activities (ALT: 12 ± 2 U/l) were significantly increased during the 1 h reperfusion period following 1 h of partial hepatic no-flow ischemia (GSSG: 19.7 ± 2.2 μM; GSH 36.9 ± 7.4 μM; ALT: 2260 ± 355 U/l). Pretreatment with 1,3-bis-(2-chloroethyl)-1-nitrosourea (40 mg BCNU/kg), which inhibited glutathione reductase activity in the liver by 60%, did not affect any of these parameters. Biliary GSSG and GSH efflux rates were reduced and the GSSG-to-GSH ratio was not altered in controls and BCNU-treated rats at any time during ischemia and reperfusion. A 90% depletion of the hepatic glutathione content by phorone treatment (300 mg/kg) reduced the increase of plasma GSSG levels by 54%, totally suppressed the rise of plasma GSH concentrations and increased plasma ALT to 4290 ± 755 U/l during reperfusion. The data suggest that hepatic glutathione serves to limit ischemia/reperfusion injury as a source of extracellular glutathione, not as a cofactor for the intracellular enzymatic detoxification of reactive oxygen species.

KEY WORDS: Reactive oxygen, glutathione, glutathione disulfide, liver.

INTRODUCTION

A role for reactive oxygen species (ROS) in the pathogenesis of ischemia/reflow injury has been suggested for the liver and other organs (for review: [1]). This view is based on the protective effects of pharmacological interventions which reduce the formation or accelerate the metabolism of ROS.[2,3] However, the exact role of reactive oxygen remained unclear.

Our own studies in the isolated blood-free perfused liver demonstrated that the quantitaties of ROS formed intracellularly were insufficient to exceed endogenous defense mechanisms during reperfusion after up to two hours of ischemia.[4] When ROS were generated chemically with diquat, the postischemic liver detoxified several orders of magnitude more ROS than were formed during reperfusion alone, without additional cell damage.[4] Further, no evidence for lipid peroxidation was detected in postischemic livers.[5,6] A significant increase of ROS release from intracellular sources such as mitochondria and xanthine oxidase was observed only during reoxygenation after severe hypoxic injury of the liver.[7,8] But even under these dramatic conditions that resulted in a significant loss of intracellular glutathione, ROS were still detoxified

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and further cell damage was drastically reduced during reoxygenation.[7,8] These data demonstrate a considerable resistance of hepatocytes against intracellular oxidant stress.[4,7,8] On the other hand, a dramatic increase of the plasma concentration of glutathione disulfide (GSSG)[9,10] correlated with the accumulation of neutrophils in the postischemic liver,[9] indicating a significant oxidant stress during reperfusion *in vivo*. Although GSSG is mainly exported into bile,[11] the biliary efflux of GSSG was not elevated in this no-flow ischemia model.[9] These results are consistent with the hypothesis that neutrophils and/or resident macrophages (Kupffer cells) may be the source of ROS formation. However, it is not clear whether ROS oxidize plasma GSH directly or are detoxified intracellularly (erythrocytes, endothelial cells) and then partially exported into the plasma. Further, it is unknown whether ROS contribute directly to the damage and if GSH of hepatic origin, when released into the plasma, can protect significantly against reperfusion damage.

This study presents evidence that reactive oxygen directly oxidizes GSH in the extracellular space and that GSH release from hepatocytes attenuate reperfusion injury in the liver.

MATERIALS AND METHODS

Animals

Fed male Fischer-344 rats (250–300 g; Harlan Sprague-Dawley, Inc. Houston, Texas) were used in all experiments. 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) (BiCNU®, Bristol-Myers Comp., Evansville, Indiana) was dissolved in corn oil (20 mg/ml) and injected intraperitoneally (40 mg BCNU/kg body wt.) 15 h prior to the experiment. 2,6-Dimethyl-2,5-heptadien-4-one (phorone; Aldrich Chemical Comp., Milwaukee, Wisconsin) was diluted in corn oil and injected ip (300 mg/kg body wt.) 1 h prior to ischemia.

Experimental Protocols

All animals were anesthetized with pentobarbital (50 mg/kg body wt.; ip) and the carotid artery was cannulated with PE-50 tubing. In some experiments the common bile duct was cannulated additionally with PE-10 tubing. Body temperature was maintained at 37.5 ± 0.5°C by a heating lamp. Blood samples of 400 μl were collected at different times during the experiment and the volume of the blood was replaced by saline. An aliquot (200 μl) of the blood was mixed immediately with 10 mM N-ethylmaleimide (NEM) in 100 mM potassium phosphate buffer (pH 6.5) for determination of GSSG.[12] The remaining blood was centrifuged for 1 min, an aliquot of the plasma was pipetted into 3% sulfosalicylic acid (SSA) for determination of total soluble glutathione and the rest of the plasma was used for determination of alanine aminotransferase (ALT) activity. Bile was collected in preweighed tubes containing 3% SSA. In ischemia experiments, the blood flow to the median and left lateral hepatic lobe was occluded with an arterial clamp for 1 h. Reperfusion was initiated by removal of the clamp. In other experiments tert-butylhydroperoxide (1 mmol tBHP/kg body wt.) was injected ip as a single dose. At the end of the experiment a part of a non-ischemic and a postischemic lobe was freeze-clamped and stored in liquid nitrogen.

Methods

Total soluble glutathione (GSH and GSSG) and GSSG was measured in bile, plasma, and acidic homogenate from freeze-clamped livers by the method of Tietze as described in detail.[4,11] ALT was determined in plasma with Sigma Test Kit DG 159-UV and is expressed as IU/l. Hepatic glutathione reductase activity was determined in the postmitochondrial supernatant of a 20% homogenate (w/w). An aliquot of the supernatant was added to 1 ml of 250 mM potassium phosphate buffer (pH 7.0), containing 250 μM NADPH and 1.5 μM FAD. The mixture was incubated at 37°C for 5 min and then the reaction was started with the addition of GSSG (1.5 mM) and the consumption of NADPH was recorded at 340 nm.

RESULTS

Pretreatment of rats with BCNU *in vivo* reduced hepatic glutathione reductase (GR) activity by 61%, from 75.5 $\pm$ 3.3 mU/mg protein to 29.4 $\pm$ 5.5 mU/mg protein ($n = 6$; $p < 0.05$). In order to verify that this pretreatment will increase GSSG release from the liver as a response to an oxidative challenge, the animals received a bolus injection of tBHP (1 mmol/kg; ip). Basal biliary GSSG efflux rates (2.47 $\pm$ 0.16 nmol GSH-eq./min/g liver wt.) increased immediately in untreated animals, reaching a maximum (56.85 $\pm$ 5.67) within the first 5 min and returned to control values within 15 min. BCNU-pretreated animals showed a similar temporal pattern but significantly higher GSSG efflux rates (basal: 5.45 $\pm$ 0.87; $p < 0.05$; max.: 96.88 $\pm$ 17.56; $p < 0.05$). The total amount of GSSG released into bile after tBHP was 355 $\pm$ 29 nmol GSH-eq./g liver wt. (controls) and 763 $\pm$ 114 (BCNU; $p < 0.05$). Basal plasma GSSG concentrations (C: 1.65 $\pm$ 0.22 μM; BCNU: 2.27 $\pm$ 0.27) also increased dramatically in both groups 5 min after tBHP (C: 60.3 $\pm$ 8.9; BCNU: 122.1 $\pm$ 22.8; $p < 0.05$, C versus BCNU). These results demonstrate that BCNU treatment enhanced significantly hepatic GSSG efflux rates as well the total amount of GSSG release as a response to an intracellular oxidant stress. To test if the postischemic increase of plasma GSSG was caused by enhanced intracellular GSSG formation and release, control and BCNU-treated animals were subjected to 1 h normothermic no-flow ischemia followed by 1 h of reperfusion. As shown in Figure 1, the concentration of GSSG and GSH in plasma did not change during ischemia but both parameters increased severalfold during reperfusion. No significant difference in plasma GSSG or GSH was observed between controls and BCNU-treated animals at any time during either ischemia or reperfusion. Likewise there was no significant difference in hepatocellular damage between these groups as reflected by a similar rise of the aminotransferase activities during the reperfusion period (Figure 1). BCNU induced a choleresis and an enhanced efflux of biliary GSH and GSSG (Table I). All biliary parameters declined significantly in both experimental groups during iscemia and partially recovered only in controls during the reperfusion period. The biliary efflux of GSSG did not increase above basal values and the GSSG-to-GSH ratio did not change either in controls or in BCNU-treated rats during ischemia and reperfusion (Table I). The GSSG content in the postischemic liver lobes of controls increased only by 30% during reperfusion when compared with the nonischemic lobes (0.21 $\pm$ 0.02 nmol GSH-eq./mg protein). Slightly higher basal GSSG levels were measured in livers of BCNU treated animals (0.35 $\pm$ 0.03; $p < 0.05$). A similar 30% increase was seen in the postischemic lobes.

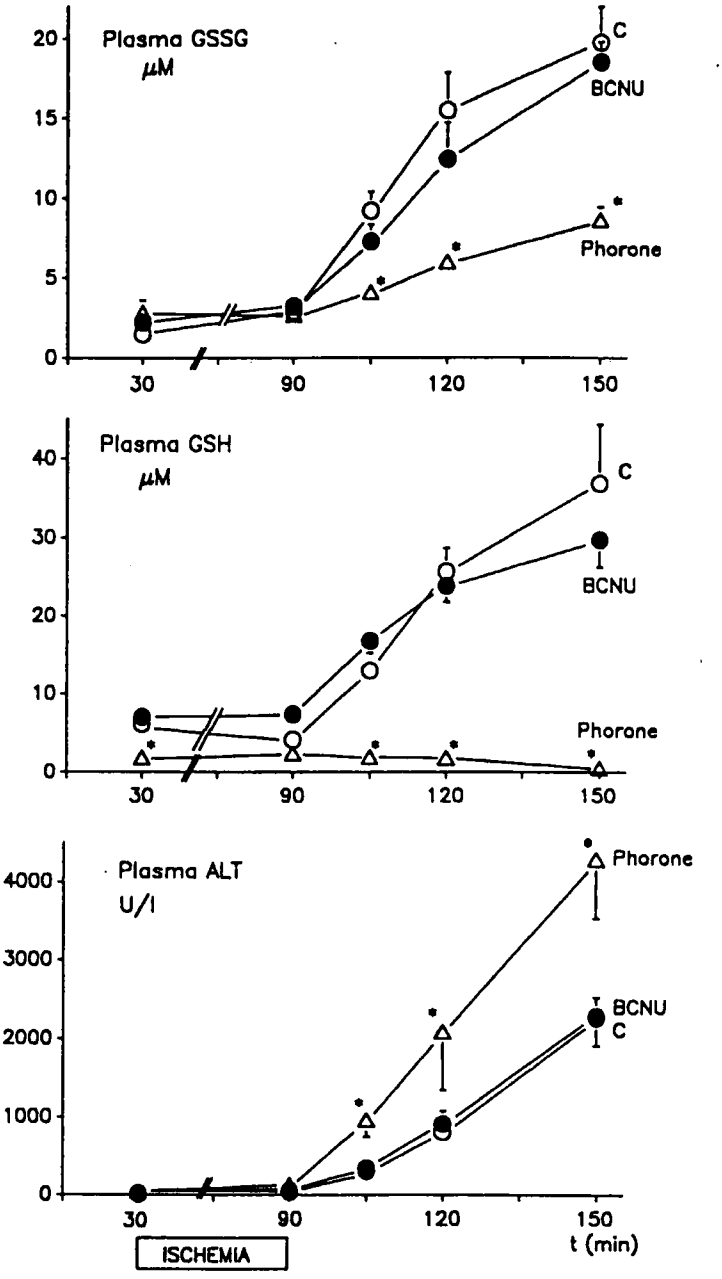

FIGURE 1 Plasma concentrations of GSSG (given as GSH-equivalents) and GSH and plasma alanine aminotransferase activities (ALT) during hepatic ischemia (60 min) and reperfusion. All values are given as mean ± S.E. (n = 6 animals). Controls (C) are compared with animals pretreated with BCNU (40 mg/kg; ip) or phorone (300 mg/kg; ip). *$p < 0.05$ (C vs. BCNU or phorone).

TABLE I
Biliary Glutathione Efflux in Controls and BCNU-treated Rats

	Pre-Ischemia[4]	Ischemia[4]	Post-Ischemia[4]
Bile Flow[1]			
C	2.20 ± 0.19	0.35 ± 0.15	0.94 ± 0.47
BCNU	3.56 ± 0.29*	0.69 ± 0.16*	0.95 ± 0.14
GSSG[2]			
C	1.36 ± 0.79	0.13 ± 0.07	0.38 ± 0.13
BCNU	6.60 ± 0.18*	0.74 ± 0.20*	0.76 ± 0.26
GSH[2]			
C	4.75 ± 0.74	0.35 ± 0.20	1.05 ± 0.14
BCNU	11.49 ± 0.36*	1.27 ± 0.28*	1.17 ± 0.52
GSSG %[3]			
C	22	27	27
BCNU	37	37	39

[1]Data are expressed as mean ± S.E. in μl/min/g liver wt (n = 3 experiments).
[2]Data are given as nmol GSH-equivalents/min/g liver wt.
[3]GSSG efflux rates are given as per cent of the total glutathione efflux in bile.
[4]Bile was collected for 15 min before ischemia (Pre-Ischemia), during 60 min of hepatic no-flow ischemia (Ischemia) and during the first 15 min of reperfusion (Post-Ischemia).

These results suggest tissue GSH released into the plasma could be directly oxidized to GSSG in the vascular compartment by reactive oxygen. To test this hypothesis, the hepatic glutathione content was depleted by phorone.[13] A dose of 300 mg phorone/kg reduced hepatic GSH from 32.2 ± 2.0 nmol/mg protein to 1.9 ± 0.2 within 1 h. As shown in Figure 1, phorone pretreatment significantly reduced the increase of plasma GSSG and totally suppressed the increase of plasma GSH during reperfusion. Thus, in phorone-treated animals plasma glutathione was almost totally (95%) oxidized to GSSG after 1 h reperfusion while only 35–40% of the plasma glutathione concentration in controls and BCNU-treated animals was present as GSSG. As a result, phorone treatment exacerbated reperfusion injury (Figure 1).

DISCUSSION

The objective of this study was to test the hypothesis that ROS, formed in the vasculature, directly oxidize GSH and that intracellular GSH, when released into the plasma, can provide a significant protection against hepatic reperfusion injury. Indirect evidence for the extracellular generation of ROS came from ischemia experiments with the isolated blood-free perfused liver, where no evidence was found for a significant intracellular oxidant stress during reperfusion.[4] In the *in vivo* model, the selective increase of the GSSG concentration in plasma suggested an oxidant stress outside of hepatocytes[9,10] since GSSG is preferentially (80–85%) released into bile.[11] Impairment of bile formation can lead to an enhanced release of GSSG into plasma, however, intracellular oxidant stress will always cause a significant increase of the biliary GSSG efflux rate even under severe cholestasis.[11] The BCNU experiments presented in this paper strongly support the idea that no enzymatic detoxification of ROS took place during reperfusion. BCNU is an alkylating agent which causes an irreversible inhibition of glutathione reductase (GR).[14] Since the efflux of GSSG is a

very sensitive indicator of the activity of glutathione peroxidase[15] and GR is the rate-limiting step of the glutathione redox cycle, inhibition of GR during an oxidant stress should result in a further enhancement of GSSG release. As demonstrated by the experiments in which tBHP was administered as an oxidant stress, BCNU pretreatment caused a significantly greater increase of biliary and plasma GSSG and considerably delayed the return to basal values. The fact, that BCNU treatment did not change plasma concentration of GSSG and GSH and did not exacerbate hepatocellular damage during ischemia aqnd reperfusion suggest that the intracellular glutathione redox cycle was not involved in the formation of GSSG. Thus, the oxidizing species was neither generated nor detoxified intercellularly. Since BCNU treatment inhibits GR not only in hepatocytes but also in various other cells, including erythrocytes and endothelial cells,[14,16] these experiments also do not support the hypothesis that a relevant portion of extracellarly generated ROS was detoxified enzymatically inside vascular cells. These results imply that glutathione is oxidized non-enzymatically in the extracellular space. Since the spontaneous oxidation of GSH in plasma is relatively slow[17] and glutathione disappears from blood with a half-life of less than 2 min,[18,19] GSSG formation must be catalyzed by an oxidant. The identity of the oxidizing species is not known at present, but hydrogen peroxide and hypochlorous acid are potential oxidants formed by neutrophils (PMNs) and macrophages. PMNs were shown to accumulate in reperfused tissued after ischemia[9,20,21] and the liver contains a large number of resident macrophages (Kupffer cells). Further experiments are necessary to elucidate the relative importance of these cells for the vascular oxidant stress and reperfusion injury.

A controversial issue is whether ROS directly cause reperfusion damage. Treatment with phorone depleted the hepatic glutathione content and consequently reduced not only the physiological GSH efflux from the liver but also the additional GSH release due to cell swelling. As a result, the increase of the plasma GSSG concentration was significantly attenuated. Since phorone also enhanced reperfusion damage, it is concluded that ROS contribute either directly or indirectly at least to the additional damage. These data confirm the importance of hepatic glutathione levels in hepatic iscehmia/reperfusion injury,[22] however, not as a cofactor of glutathione peroxidase but as a source of plasma GSH and thus an extracellular reductant of ROS. This concept would also explain the beneficial effect of extraccellular superoxide dismutase and catalase[2,3] on ischemia/reperfusion injury and recent evidence for lipid peroxidation in the endothelial cell and Kupffer cell fraction of the postischemic liver.[23] Although ROS derived from PMNs and Kupffer cell can cause cell damage, the release of proteolytic enzymes from PMN may also be involved.[24]

In summary, this paper presents evidence for the extracellular oxidation of GSH as an important mechanism for limiting reperfusion injury *in vivo*. The hepatic glutathione content is a crucial factor in the pathogenesis as a source of extracellular glutathione not as a cofactor for the intracellular enzymatic detoxification of ROS.

Acknowledgements

This work was supported by USPHS grant RR-05425 and NIH grants GM 42957 and GM 34120. The author thanks Michael Fisher and Brad Black for their expert technical assistance.

References

1. J.M. McCord (1985) Oxygen-derived free radicals in post-ischemic tissue injury. *New England Journal of Medicine*, **312**, 159–163.

2. D. Adkison, M.E. Hoellwarth, J.N. Benoit, D.A. Parks, J.M. McCord and D.N. Granger (1985) Role of free radicals in ischemia-reperfusion injury to the liver. *Acta Physiologica Scandinavica Suppl.*, **548**, 101–107.
3. S.L. Atalla, L.H. Toledo-Pereyra, G.H. MacKenzie and J.P. Cederna (1985) Influence of oxygen-derived free radical scavengers on ischemic livers. *Transplantation*, **40**, 584–590.
4. H. Jaeschke, C.V. Smith and J.R. Mitchell (1988) Reactive oxygen species during ischemia-reflow injury in isolated perfused rat liver. *Journal of Clinical Investigation*, **81**, 1240–1246.
5. E.H. Silver and S. Szabo (1983) Role of lipid peroxidation in tissue injury after hepatic ischemia. *Experimental and Molecular Pathology*, **38**, 69–76.
6. J.R. Mitchell, C.V. Smith, H. Hughes, M.L. Lenz, H. Jaeschke, S. Shapell, L. Michael and M.L. Entman (1987) No evidence for reactive oxygen damage in ischemia/reflow injury. *Transactions of the Association of American Physicians*, C, 54–61.
7. H. Jaeschke, C.V. Smith and J.R. Mitchell (1988)) Hypoxic damage generates reactive oxygen species in isolated perfused rat liver. *Biochemical and Biophysical Research Communications*, **150**, 568–574.
8. H. Jaeschke and J.R. Mitchell (1989) Mitochondria and xanthine oxidase both generate reactive oxygen species after hypoxic damage in isolated perfused rat liver. *Biochemical and Biophysical Research Communications*, **160**, 140–147.
9. H. Jaeschke, C.V. Smith, H. Hughes and J.R. Mitchell (1988) Leukocyte infiltration and oxidative stress in ischemia/reflow injury. *Hepatology*, **8**, 1250 (abstr.)
10. J. Metzger, S.P. Dore and B.H. Lauterburg (1988) Oxidant Stress during reperfusion of ischemic liver: No evidence for a role of xanthine oxidase. *Hepatology*, **8**, 580–584.
11. H. Jaeschke and J.R. Mitchell (1990) The use of isolated perfused organs in hypoxia and ischemia/reflow oxidant stress. *Methods in Enzymol.*, **186**, 752–759.
12. J.D. Adams, B.H. Lauterburg and J.R. Mitchell (1983) Plasma glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress. *Journal of Pharmacology and Experimental Therapeutics*, **227**, 749–754.
13. H. Jaeschke, C. Kleinwaechter and A. Wendel (1987) The role of acrolein in allyl alcohol-induced lipid peroxidation and liver cell damage in mice. *Biochemical Pharmacology*, **36**, 51–57.
14. H. Fisher and T. Ahmad (1977) Severe generalized glutathione reductase deficiency after antitumor chemotherapy with BCNU. *Journal of Laboratory Clinical Medicine*, **89**, 1080–1091.
15. H. Sies, C. Gerstenecker, H. Menzel and L. Flohe (1972) Oxidation in the NADP system and release of GSSG from hemoglobin-free perfused rat liver during peroxidatic oxidation of glutathione by hydroperoxides. *FEBS Letters*, **27**, 171–175.
16. N. Suttorp, W. Toepfer and L. Roka (1986) Antioxidant defense mechanisms of endothelial cells: glutathione redox cycle versus catalase. *American Journal of Physiology*, **251**, C671–C680.
17. M. Inoue, Y. Saito, E. Hirata, Y. Morino and S. Nagase (1986) Regulation of redox states of plasma proteins by metabolism and transport of glutathione and related compounds. *Journal of Protein Chemistry*, **6**, 207–225.
18. A. Wendel and P. Cykrit (1980) The level and half-life of glutathione in human plasma. *FEBS Letters*, **120**, 209–211.
19. A. Wendel and H. Jaeschke (1982) Drug-induced lipid peroxidation in mice III: Glutathione content of liver, kidney and spleen after intravenous administration of free and liposomally entrapped glutathione. *Biochemical Pharmacology*, **31**, 3607–3611.
20. B.R. Lucchesi and K.M. Mullane (1986) Leukocytes and ischemia-induced myocardial injury. *Annual Reviews in Pharmacology and Toxicology*, **26**, 201–224.
21. D.N. Granger (1988) Role of xanthine oxidase and granulocytes in ischemia-reperfusion injury. *American Journal of Physiology*, **255**, H1269–1275.
22. E. Jennische (1984) Possible influence of glutathione on postischemic liver injury. *Acta Pathologica, Microbiologica et Immunologica Scandinavica*, **92**, 55–64.
23. T.R. Walsh, P. Rao, L. Makowka and T.E. Starzl (1989) Lipid peroxidation is a non-parenchymal cell event with reperfusion after prolonged liver ischemia. *FASEB Journal*, **3**, A1000 (abstr.).
24. P. Mavier, A.M. Preaux, B. Guigui, M.C. Lescs, E.S. Zafrani and D. Dhumeaux (1988) *In vitro* toxicity of polymorphonuclear neutrophils to rat hepatocytes: evidence for a proteinase-mediated mechanism. *Hepatology*, **8**, 254–258.

Accepted by Prof. G. Gzapski