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CARBON TETRACHLORIDE INCREASES SINUSOIDAL EFFLUX OF REDUCED AND OXIDIZED GLUTATHIONE IN RATS

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Abstract—To elucidate the significance of the changes in plasma glutathione concentrations associated with carbon tetrachloride (CCl_4)-induced liver damage, the changes in the concentrations of reduced (GSH) and oxidized glutathione (GSSG) in plasma as well as in the liver were investigated in rats. In the liver, the concentration of GSH decreased, and that of GSSG increased 24 hr after the intraperitoneal administration of CCl_4. In the right atrial plasma, the concentration of both GSH and GSSG increased. The GSH/GSSG ratio in the plasma decreased as did that in the liver. The net sinusoidal efflux of GSH and GSSG from the liver was calculated by subtracting their concentrations in plasma of the infrahepatic inferior vena cava from those of the suprahepatic inferior vena cava. The net efflux of GSH and GSSG started to increase as early as 3–6 hr after CCl_4 administration, and reached a plateau 6 and 24 hr after CCl_4 administration, respectively. On the other hand, an elongation of prothrombin time and leakage of alanine aminotransferase reached a maximum 24 and 48 hr after CCl_4 administration, respectively. Vacuolization in the centri-lobular region and inflammatory infiltration started 3 and 6 hr after CCl_4 administration, respectively, and progressed for 48 hr. These results suggest that CCl_4 induced an increase in plasma concentrations of GSH as well as GSSG by increasing their efflux from the liver, and that the changes in plasma glutathione status might be a useful and sensitive marker for CCl_4-induced liver damage.

Carbon tetrachloride (CCl_4) has been widely used to elicit experimental liver damage [1]. CCl_4-induced liver damage has been thought to depend on the formation of reactive intermediates such as trichloromethyl free radical ($^{\cdot}CCl_3$) produced by the cytochrome P450 mixed function oxidase system [1]. The reactive metabolites initiate lipid peroxidation or bind covalently to lipids and proteins, resulting in destruction of membranes [1, 2]. Recently, involvement of Kupffer cells and PMNLs in the pathogenesis of CCl_4-induced liver damage has been suggested [3–5]. Therefore, CCl_4 seems to damage the liver by numerative oxidative mechanisms.

A role of glutathione in the detoxification of electrophilic metabolites of xenobiotics and reactive oxygen intermediates has been established [6, 7]. GSH/GSSG§ is the most abundant intracellular redox system, and the maintenance of thiol/disulfide redox states regulates many cellular processes [8]. The liver is not only the major site of detoxification reactions but also the major source of extracellular glutathione [9]. Recently, it has been suggested that an increase in plasma concentrations of GSSG may be a useful marker for oxidative stress [7, 10, 11]. The effects of CCl_4 administration on hepatic glutathione status have been extensively studied, and it has been suggested that hepatic GSH plays an important role in ameliorating CCl_4-induced liver damage [12–15]. However, its effects on plasma glutathione concentrations are still unclear. We studied the changes in GSH and GSSG concentrations in plasma as well as in the liver after the administration of CCl_4, and compared them with those in PT, serum ALT activity and light-microscopic findings.

MATERIALS AND METHODS

Animals. All animal experiments were conducted in accordance with the Kyushu University guidelines for the care and the use of laboratory animals. Seven-week-old, male Wistar rats weighing about 200 g were used. Food deprivation was started 24 hr before the injection of CCl_4 to make the rats susceptible to CCl_4 [11, 12, 15, 16], and continued until blood samples were taken. Water was offered *ad libitum*. A 1 mL/kg dose of CCl_4 dissolved in olive oil (50% solution) was injected intraperitoneally. Control rats were given olive oil, 0.5 mL/kg. Blood and liver samples were taken under anesthesia with

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§ Abbreviations: GSH reduced glutathione; GSSG, oxidized glutathione; PT, prothrombin time; ALT, alanine aminotransferase; PMNL, polymorphonuclear leukocyte.

diethyl ether at the time indicated in the text or legends for figures. CCl_4 was purchased from Nakarai Chemical (Kyoto, Japan).

Assays. Assays for free GSH and GSSG in plasma and in the liver were done according to the method by Shibata *et al.* [17]. Fresh blood samples were immediately mixed with EDTA, 2 mM. Perchloric acid at a final concentraion of 0.5 N was immediately added to the plasma, followed by centrifugation at 15,000 *g* for 1 min on Himac CR15-D centrifuge (Hitachi, Tokyo, Japan) at 4°. Liver samples (approximately 1 g) were immediately immersed into liquid nitrogen and stored at −70°. The frozen samples were homogenized in a polytron and Potter–Elvehjem homogenizer in the presence of perchloric acid, 0.5 N, then centrifuged. The deproteinized plasma and the liver extract were neutralized with sodium acetate buffer, pH 4.2 and KOH, followed by centrifugation. The resultant supernatant was introduced into a Shimpack CLC-ODS reverse-phase HPLC column (Hitachi, 150 mm in length 6 mm in diameter). The column was eluted with sodium phosphate, 100 mM, pH 2.3, at a flow rate of 1 mL/min. GSH was quantitated by an Amperometer Σ875 (Irica Instruments Inc., Kyoto, Japan) at an electrode voltage of 0.6 V. GSSG was quantitated by a SPD-6A ultraviolet spectrophotometric detector (Shimadzu Corp., Kyoto, Japan) at a wavelength of 220 nm. GSH and GSSG for the standards were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Serum ALT activity and PT were measured with an Ektachem DT60 analyser (Eastman Kodak, Rochester, N.Y., U.S.A.) and the Thromboplastin·C (Baxter Diagnostics, Miami, FL, U.S.A.), respectively.

Histological examination of the liver. Samples of liver tissue were fixed in 10% formalin and embedded in paraffin for light-microscopic examination. Sections of liver tissue, 5 μm thick, were stained with hematoxylin and eosin.

Statistical analysis. All results are expressed as mean ± SD. Statistical analysis of the data obtained in each group was performed using the Student's *t*-test. $P < 0.05$ was considered statistically significant.

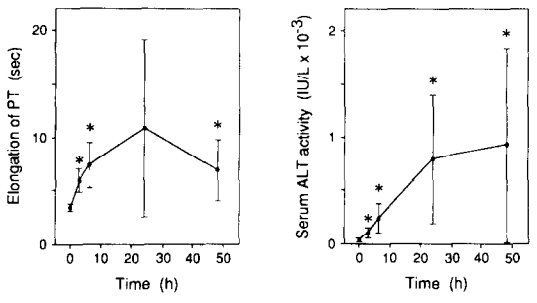


Fig. 1. Time course of the changes in the net sinusoidal efflux of GSH and GSSG after CCl_4 injection in starved rats. Rats were divided into five groups (N = 7–11 per group). CCl_4, 0.5 mL/kg, was administered i.p. to 7-week-old, male Wistar rats starved for 24 hr. Food deprivation was continued, while water was offered *ad libitum*. Blood samples and the liver were taken under anesthesia 0, 3, 6, 24 and 48 hr after CCl_4 injection. The net efflux was calculated by subtracting GSH and GSSG concetrations in plasma obtained from the infrahepatic inferior vena cava from those obtained from the suprahepatic inferior vena cava. *$P < 0.05$ vs coincident values at 0 hr.

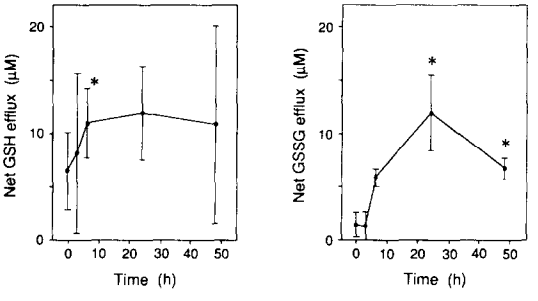


Fig. 2. Time course of the elongation of PT and the increase in serum ALT activity after CCl_4 injection in rats. Blood samples taken from the suprahepatic inferior vena cava in Fig. 1 were analysed. *$P < 0.05$ vs coincident values at 0 hr.

RESULTS

Intraperitoneal administration of CCl_4, 0.5 mL/kg induced the following changes in GSH and GSSG concentrations in the liver and in the plasma over a period of 24 hr (N = 7–11). In the liver, the concentration of GSH decreased from 5.8 ± 1.2 to 4.5 ± 1.5 μmol/g of liver ($P < 0.0001$). This resulted in a decrease in the hepatic GSH/GSSG ratio from 18.4 ± 4.8 to 6.2 ± 2.3 ($P < 0.05$). In the plasma taken from the right atrium, both the concentration of GSH (from 14.8 ± 3.6 to 21.2 ± 7.2 μM, $P < 0.05$) and GSSG (from 0.6 ± 0.2 to 3.6 ± 2.6 μM, $P < 0.05$) increased after the injection. As the increase in the concentration of GSSG was greater than that in GSH, the GSH/GSSG ratio in the plasma decreased from 25.3 ± 8.0 to 12.0 ± 10.7 ($P < 0.0001$).

To examine whether the observed increase in the plasma GSH and GSSG concentrations was caused by the increase in an influx from the liver, we measured the net efflux of GSH and GSSG by subtracting their concentrations in plasma taken at the infrahepatic inferior vena cava from those taken at the suprahepatic inferior vena cava. The control values of the net efflux of GSH and GSSG were 6.5 ± 3.9 (N = 7) and 1.5 ± 2.2 μM (N = 4), respectively (Fig. 1). The net efflux of GSSG started to increase 6 hr after the injection, and reached its maximum 24 hr after the injection. The net efflux of GSH started to increase 3 hr after the injection, and reached a plateau 6 hr after the injection.

An elongation of PT started to occur 3 hr after CCl_4 injection ($P < 0.005$), and reached its maximum 24 hr after the injection (Fig. 2). Serum ALT activity started to increase 3 hr after CCl_4 injection ($P < 0.005$), and reached a plateau 24–48 hr after the injection.

Light-microscopic examination of liver tissues

showed that vacuolar degeneration and inflammatory infiltration started to occur 3 and 6 hr after CCl_4 injection, respectively (Fig. 3). Vacuolar degeneration progressed up to 48 hr after the injection, and massive necrosis of hepatocytes was observed 24 hr after the injection. These changes were more marked in the centri-lobular region than in the peri-portal region. Inflammatory infiltration also progressed up to 48 hr after the injection.

DISCUSSION

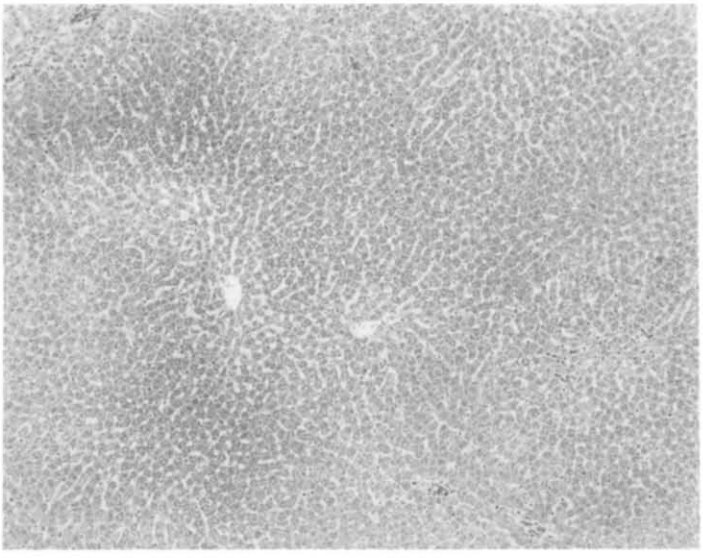
It has been widely accepted that, when the rate of oxidation of GSH to GSSG exceeds the capacity of GSSG reductase, GSSG is actively transported out of the cell to maintain the intracellular GSH/GSSG ratio [7]. We have found that GSH as well as GSSG increases in plasma after the administration of CCl_4, 0.5 mL/kg in starved Wistar rats. We have also shown that these changes are partly explained by an increase in the net sinusoidal efflux of GSH and GSSG. The net efflux of GSH seems to increase earlier than that of GSSG. Furthermore, the increase in the net efflux of GSH reaches a plateau or its maximum earlier than GSSG, an elongation of PT and an increase in serum ALT activity.

The possibility that the observed efflux was due to leakage secondary to plasma membrane damage rather than an active process could be ruled out because of the different time courses and magnitude of the changes in GSH efflux, GSSG efflux and serum ALT activity. Another possibility, that the degradation of circulating GSH was suppressed by an inactivation of hepatic γ-glutamyltransferase due to damage of the hepatocyte plasma membrane [18], can be ruled out for the same reasons. Inactivation of renal γ-glutamyltransferase seems unlikely, because light-microscopic examination showed no change in the kidney (not shown). The increased concentration of hepatic GSSG, the decreased hepatic GSH/GSSG ratio and the increased efflux of GSSG indicate that the liver was under oxidative stress [7, 10, 11]. However, the increased efflux of GSSG does not necessarily imply that GSSG is transported from hepatocytes. Because hepatic endothelial cells have been shown to be damaged by CCl_4 [5], it is possible that GSH and GSSG may be released from these cells. Furthermore GSSG might be formed extracellularly in sinusoids by activated Kupffer cells and PMNLs [19]. However, GSSG efflux reached its maximum much earlier than the infiltration of PMNLs, suggesting that activated PMNLs might not be involved in the observed increase in GSSG efflux.

Adams *et al.* [20] observed that the GSSG concentration in femoral venous plasma increased 30 min after the intraperitoneal injection of CCl_4, about 1.0 mL/kg in non-starved, male Sprague–Dawley rats weighing 250–350 g, but they failed to detect any increase in plasma GSH concentration using enzymatic analysis. The reasons for the discrepancy between their results and ours are unclear, but the difference in feeding seems to be the most likely. It is well known that starvation deranges the tissue antioxidant state, including a decrease in GSH concentrations both in the liver and in plasma [11, 16]. We have reported that the administration of CCl_4, 0.5 mL/kg in non-starved rats did not affect plasma GSH concentration after 24 hr, whereas it did increase plasma GSH concentration in rats starved for 24 hr [15]. Furthermore, liver damage judged by an increase in serum ALT activity and light microscopic examination was much more severe in starved rats than in non-starved ones, leading to the speculation that starvation induces changes in energy and glutathione metabolism resulting in impaired liver defence against CCl_4 and repair mechanisms [15]. Adams *et al.* [20] used a different analytical method for GSH determination: they used enzymatic analysis; we employed amperometric detection combined with separation by HPLC. The latter is more sensitive and reproducible than the former [17].

The changes in the sinusoidal efflux of GSH under pathological conditions have not been given much attention until recently, because plasma GSH concentrations have been thought to reflect mainly hapatic GSH concentrations [11, 21], and the increase in plasma GSSG concentrations alone has been emphasized as a useful marker for oxidative stress *in vivo* [7, 10, 11, 20–25]. Recently, there have appeared several reports showing an increase in plasma concentrations of GSH as well as GSSG under pathological conditions in which oxidative mechanisms have been thought to be involved [19, 24–29]. The sinusoidal efflux of GSH has been shown to increase in rats which ingest ethanol chronically [26]. Jaeschke and Farhood [19] have demonstrated that ischemia/reperfusion of rat liver induces an increase not only in GSSG but also in GSH concentrations in plasma, which precedes an increase in plasma ALT activity. Metzger *et al.* [24] have also observed an increase in perfusate GSH concentrations by ischemia/reperfusion using perfused rat liver, but they focused on an increase in GSSG. Adams *et al.* [20] have observed an increase, although not significant, in plasma concentrations of GSH in rats injected intraperitoneally with *t*-butyl hydroperoxide. We have obtained similar results in rats given small simultaneous doses of endotoxin and D-galactosamine intraperitoneally (submitted for publication). The increased efflux of GSH despite a decrease in hepatic GSH concentration must have some pathophysiological significance. Circulating GSH may be an important determinant of the plasma thiol/disulfide ratio and may play a critical role in regulating functions of serum proteins and receptors [8]. It seems possible that an increased efflux of GSH from the liver plays a role in maintaining the plasma GSH/GSSG ratio. The difference in the time course between the GSH and GSSG efflux, however, suggests the involvement of other factors in the increase in the sinusoidal efflux of GSH in spite of the decreased hepatic GSH concentration. The low concentrations of hepatic GSH do not necessarily mean production of hepatic GSH is suppressed [9]. A decrease in hepatic GSH concentration seems to be explained by oxidation to GSSG, an increased efflux, an adduct formation with CCl_4 metabolites [13], and protein mixed disulfide formation [14].

(a)



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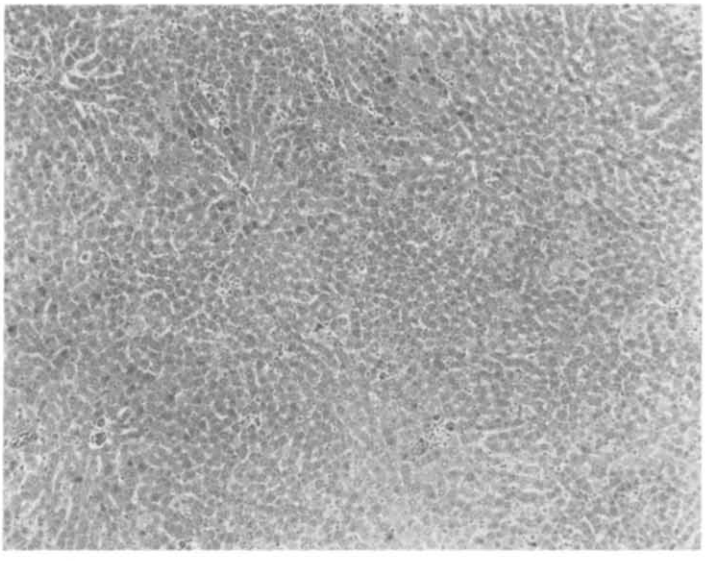

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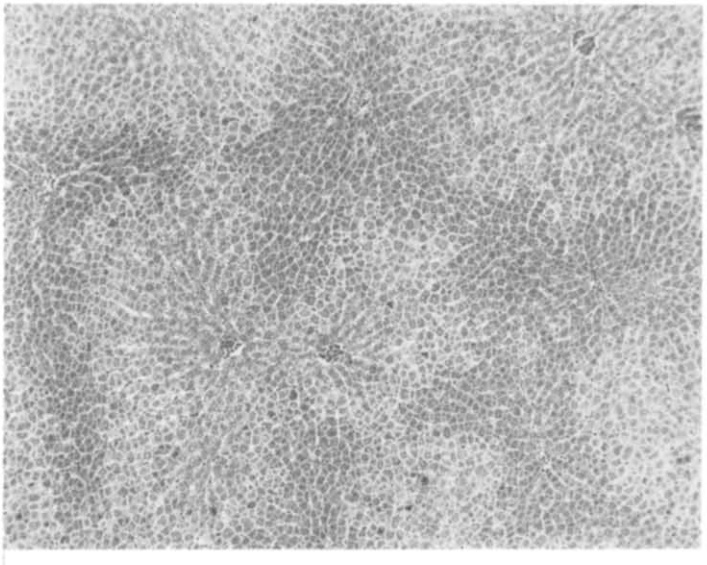

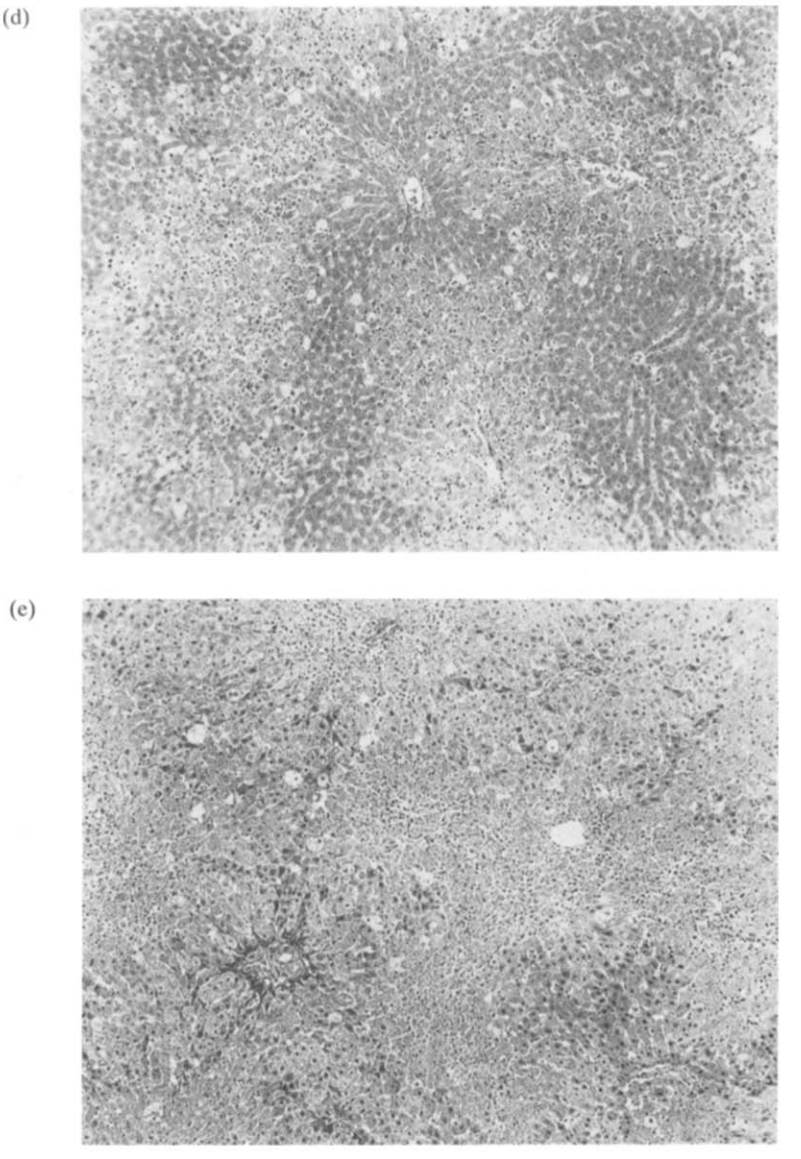

Fig. 3. Time course of the histological changes in the liver after CCl_4 injection in rats. The liver tissues taken were fixed in 10% formalin followed by staining with hematoxylin and eosin. Figures 3a–e were taken 0, 3, 6, 24 and 48 hr after CCl_4 injection, respectively. (Magnification, ×70.)

Although oxidative stress in the liver has been shown to induce an increase in biliary efflux of GSH and GSSG, an increase in biliary excretion of GSH has not been reported in rats given CCl_4 [30].

The GSH efflux associated with oxidative stress might occur in organs other than the liver. Ischemia/reperfusion of the heart has been shown to increase both GSH and GSSG concentrations in the perfusate [27] or in the coronary sinus plasma [28]. Ischemia/reperfusion of rat intestine [25] and intravenous administration of endotoxin in rats [29] have also been shown to increase both GSH and GSSG concentrations in plasma, although the source of GSH and GSSG has not been identified. Because glutathione may play a role in the cellular uptake of amino acids (γ-glutamyl cycle) and may be a reservoir of cysteine [8, 11], it should be elucidated whether an increase in a sinusoidal efflux of GSH is associated with oxidative stress in organs other than the liver.

Another observation in the present paper is that an increase in the net sinusoidal efflux of GSH and GSSG reached its maximum earlier than the changes in PT, serum ALT activity and light-microscopic changes. This is also the case in rats exposed to small doses of endotoxin and D-galactosamine (submitted for publication). The clinical relevance of measuring plasma GSH and GSSG in various conditions induced especially by oxidative mechanisms, and the biochemical basis for the increased sinusoidal efflux of GSH and GSSG should be elucidated. Quantitation of the increased efflux of GSH and GSSG is also required, because we did

not measure the blood flow in the suprahepatic and the infrahepatic inferior vena cava.

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REFERENCES

1. Reckagel RO, Glende EA Jr, Dolak JA and Waller RL, Mechanisms of carbon tetrachloride toxicity. *Pharmacol Ther* **43**: 139–154, 1989.
2. Becker E, Messner B and Berndt J, Two mechanisms of CCl_4-induced fatty liver: lipid peroxidation or covalent binding studied in cultured rat hepatocytes. *Free Radical Res Commun* **3**: 299–308, 1987.
3. Sipes IG, Elsisi AE, Sim WW, Mobley SA and Earnest DL, Role of reactive oxygen species secreted by activated Kupffer cells in the potentiation of carbon tetrachloride heptotoxicity by hypervitaminosis A. In: *Cells of the Hepatic Sinusoid* (Eds. Wisse E, Knook DL and Decker K), Vol. II, pp. 376–379. Kupffer Cell Foundation, Rijswijk, 1989.
4. Armendariz-Borunda J, Seyer JM, Postlethwaite AE and Kang AH, Kupffer cells from carbon tetrachloride-injured rat livers produce chemotactic factors for fibroblasts and monocytes: the role of tumor necrosis factor-α. *Hepatology* **14**: 895–900, 1991.
5. Sato N and Yoshihara H, Ischemic liver disease. *Kan Tan Sui (Japan)* **19**: 285–291, 1989.
6. Shan X, Aw TY and Jones DP, Glutathione-dependent protection against oxidative injury. *Pharmacol Ther* **47**: 61–71, 1990.
7. Smith CV, Correlations and apparent contradictions in assessment of oxidant stress status *in vivo*. *Free Radical Biol Med* **10**: 217–224, 1991.
8. Gilbert HF, Molecular and cellular aspects of thiol-disulfide exchange. *Adv Enzymol* **63**: 69–172, 1989.
9. Lauterburg BH, Adams JD and Mitchell JR, Hepatic glutathione homeostasis in the rat: efflux accounts for glutathione turnover. *Hepatology* **4**: 586–590, 1984.
10. Pryor WA and Godber SS, Noninvasive measures of oxidative stress status in humans. *Free Radical Biol Med* **10**: 177–184, 1991.
11. Lauterburg BH, Smith CV and Mitchell JR, Regulation of hepatic glutathione homeostasis. In: *Drug Metabolism and Toxicity* (Eds. Mitchell JR and Horning MG), pp. 321–330. Raven Press, New York, 1984.
12. Harris RN and Anders MW, Effect of fasting, diethyl maleate, and alcohols on carbon tetrachloride-induced hepatotoxicity. *Toxicol Appl Pharmacol* **56**: 191–198, 1980.
13. Reiter R and Burk RF, Formation of glutathione adducts of carbon tetrachloride metabolites in a rat liver microsomal incubation system. *Biochem Pharmacol* **37**: 327–331, 1988.
14. Brigelius R, Muckel C, Akerboom TPM and Sies H, Identification and quantitation of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. *Biochem Pharmacol* **32**: 2529–2534, 1983.
15. Irita K, Okabe H, Sakai H, Koga A, Yamakawa M and Yoshitake J, The relationship between starvation and carbon tetrachloride-induced liver damage in the rat. *Hiroshima J Anesth* **28**: 13–23, 1992.
16. Godin DV and Wohaieb SA, Nutritional deficiency, starvation, and tissue antioxidant status. *Free Radical Biol Med* **5**: 165–176, 1988.
17. Shibata H, Furuya E and Tagawa K, Determination of glutathione by high performance liquid chromatography. *Protein Nucleic Acid Enzyme* **33**: 1392–1396, 1988.
18. Speisky H, Shackel N, Varghese G, Wade D and Israel Y, Role of hepatic γ-glutamyltransferase in the degradation of circulating glutathione: studies in the intact guinea pig perfused liver. *Hepatology* **11**: 843–849, 1990.
19. Jaeschke H and Farhood A, Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. *Am J Physiol* **260**: G355–G362.
20. Adams JD Jr, Lauterburg BH and Mitchell JR, Plasma glutathione disulfide as an index of oxidant stress *in vivo*: effects of carbon tetrachloride, dimethylnitrosamine, nitrofurantoin, metronidazole, doxorubicin and diquat. *Res Commun Chem Pathol Pharmacol* **46**: 401–410, 1984.
21. Adams JD Jr, Lauterburg BH and Mitchell JR, Plasma glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress. *J Pharmacol Exp Ther* **227**: 749–754, 1983.
22. Sies H and Summer K-H, Hydroperoxide-metabolizing systems in rat liver. *Eur J Biochem* **57**: 503–512, 1975.
23. Bartoli G and Sies H, Reduced and oxidized glutathione efflux from liver. *FEBS Lett* **86**: 89–91, 1978.
24. Metzger J, Dore SP and Lauterburg BH, Oxidant stress during reperfusion of ischemic liver: no evidence for a role of xanthine oxidase. *Hepatology* **8**: 580–584, 1988.
25. Abdalla EK, Caty MG, Guice KS, Hinshaw DB and Oldham KT, Arterial levels of oxidized glutathione (GSSG) reflect oxidant stress *in vivo*. *J Surg Res* **48**: 291–296, 1990.
26. Fernandez-Checa JC, Ooktens M and Kaplowitz N, Effect of chronic ethanol feeding on rat hepatocytic glutathione: compartmentation, efflux, and response to incubation with ethanol. *J Clin Invest* **80**: 57–62, 1987.
27. Ferrari R, Cargnoni A, Curello S, Boffa GM and Ceconi C, Effects of iloprost (ZK 36374) on glutathione status during ischaemia and reperfusion of rabbit isolated hearts. *Br J Pharmacol* **98**: 678–684, 1989.
28. Lesnefsky EJ, Dauber IM and Horwitz LD, Myocardial sulfhydryl pool alterations occur during reperfusion after brief and prolonged myocardial ischemia *in vivo*. *Circ Res* **68**: 605–613, 1991.
29. Chang S, Lauterburg BH and Voelkel NF, Endotoxin causes neutrophil-independent oxidative stress in rats. *J Appl Physiol* **65**: 358–367, 1988.
30. Lauterburg BH, Smith CV, Hughes H and Mitchell JR, Biliary excretion of glutathione and glutathione disulfide in the rat: regulation and response to oxidative stress. *J Clin Invest* **73**: 124–133, 1984.