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Mechanisms of endotoxin-induced haem oxygenase mRNA accumulation in mouse liver: synergism by glutathione depletion and protection by *N*-acetylcysteine

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In *in vitro* systems haem oxygenase-1 (HO-1) mRNA increases after exposure to agents causing oxidative stress. We lowered cellular antioxidant defence systems *in vivo* by giving mice increasing doses (0.15 g/kg–1.6 g/kg) of DL-buthionine-(*S*,*R*)-sulphoximine (BSO), a specific inhibitor of glutathione synthesis. Maximum glutathione depletion (80 %) coincided with maximum hepatic HO-1 mRNA accumulation (about 20 times), whereas with 50 % depletion, accumulation was only doubled. It has been suggested that reactive oxygen and nitrogen intermediates are involved in hepatic toxicity of endotoxin (lipopolysaccharide, LPS); LPS even at low doses [0.1 mg/kg, intraperitoneally (i.p.)] induces HO-1 mRNA about 25-fold after 1 h. Hepatic glutathione depletion (respectively 40 % and 80 %) after a low (0.3 g/kg) or a high (1.6 g/kg) BSO dose, resulted in potentiation of the HO-1 mRNA accumulation induced by LPS (0.1 mg/kg, i.p.). In the absence of BSO, *N*-acetylcysteine (NAC) (1 g/kg orally) reduced LPS-induced HO-1 mRNA accumulation to one fourth. Under the same experimental conditions *S*-adenosylmethionine (SAM) was not effective. NAC also reduced HO-1 mRNA accumulation when administered to mice in which glutathione was depleted and its synthesis blocked by BSO (1.6 g/kg). Thus reactive oxygen intermediates are likely mediators of LPS-induced HO-1 mRNA accumulation, and glutathione content appears to be one of the factors regulating this accumulation in the liver. Our findings are compatible with the theory that HO-1 induction might have a protective function *in vivo* when defence mechanisms against oxidants are challenged.

INTRODUCTION

Haem oxygenase (HO, EC 1.14.99.3) is the rate-limiting enzyme in the oxidative degradation of haem. It cleaves the haem ring at the α-methene bridge to form CO and biliverdin, which is further metabolized to bilirubin by biliverdin reductase (Tenhunen et al., 1969). HO in its inducible isoenzyme form HO-1 (Maines, 1988) is activated by oxidative stress in a wide variety of mammalian cells (Applegate et al., 1991) and this induction may have beneficial properties on account of the potent chain-breaking activity of bilirubin, the end-product of the HO-reaction (Stocker et al., 1987) and the fact that catabolism of haem may rid the cell of a potentially toxic, membrane-permeant form of iron (Gutteridge and Smith, 1988). HO-1 is induced by oxidative stress caused by treatment with agents that are oxidants or can generate active intermediates or by agents which are known to interact with or modify cellular glutathione levels (Applegate et al., 1991). U.v. A radiation (320–380 nm) and H_2O_2 caused accumulation of HO-1 mRNA and transcriptional activation of the HO-1 gene in human skin fibroblasts (Keyse and Tyrrell, 1989a; Keyse et al., 1990) and the hydroxyl radical ($OH^{\cdot}$) is a key intermediate in the induction obtained with these agents (Keyse and Tyrrell, 1989b). Buthionine sulphoximine (BSO), a glutathione-depleting compound, induced hepatic HO activity (Oguro et al., 1990), HO protein (Saunders et al., 1991) and HO-1 mRNA *in vitro* (Lautier et al., 1992) and *in vivo* in the rat brain (Ewing and Maines, 1993).

Glutathione is a tripeptide containing glycine, glutamic acid and cysteine, with many functions. It is a cofactor for many glycolytic enzymes and for enzymes involved in amino acid catabolism and conversion, and it plays a key role in the accurate formation of protein bonds. The reduced form, GSH, constitutes > 90 % of the cellular non-protein thiols and of total glutathione (glutathione) and serves as the major cellular antioxidant. GSH reacts with reactive electrophilic metabolites formed by cytochrome *P*-450-mediated reactions or with reactive oxygen intermediates (ROI) produced during cell metabolism. In addition it serves as a substrate for the glutathione-peroxidase redox system in detoxification of peroxides (Meister and Anderson, 1983; Orrenius and Moldéus, 1984). For these reasons glutathione depletion can impair the cell's defence against the toxic actions of such oxidative compounds and may lead to cell injury and death (Reed, 1990). Increasing toxicity by lowering tissue glutathione content is an indirect approach to illustrate the involvement of reactive intermediates in the mechanism of action of xenobiotics (Reed and Fariss, 1984).

It has been suggested that ROI cause tissue injury during sepsis or endotoxic shock (Ferluga and Allison, 1978; Fantone and Ward, 1982; Ghezzi et al., 1993). In the liver, treatment with endotoxin [lipopolysaccharide (LPS)] lowered GSH in rats and mice (Kunimoto et al., 1987; Sugimoto et al., 1991) and it is often associated with an increase in lipid peroxidation, one of the toxic consequences of oxidative damage (Peavy and Fairchild, 1986; Sakaguchi et al., 1981). Treating the animals with antioxidants

Abbreviations used: HO-1, haem oxygenase-1; BSO, DL-buthionine-(*S*,*R*)-sulphoximine; LPS, lipopolysaccharide; i.p., intraperitoneally; NAC, *N*-acetylcysteine, SAM, *S*-adenosylmethionine; ROI, reactive oxygen intermediates; PMN, polymorphonuclear neutrophils; NF, nuclear factor; IL, interleukin; TNF, tumour necrosis factor; γGCS, γ-glutamylcysteine synthetase.

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protected against LPS toxicity (Bernard et al., 1984; Sugino et al., 1987; Peristeris et al., 1992).

In the liver the Kupffer cells are the major target for LPS: these hepatic macrophages take up LPS by absorptive phagocytosis (Mathison and Ulevitch, 1979; Praaning-van Dalen et al., 1981). In these cells and in hepatocytes LPS activates systems that produce ROI. There is experimental evidence of increased superoxide anion (O_2^-) release from Kupffer cells (Bautista et al., 1990) and the hepatic xanthine oxidase is activated (Ghezzi et al., 1984). By spontaneous dismutation O_2^- can form H_2O_2 and in the presence of a transition metal it can react with H_2O_2 forming other ROI, like the hydroxyl anion (OH^-) and the hydroxyl radical ($OH^{\bullet}$) (McCord and Fridovich, 1978). LPS induces migration to the liver of activated polymorphonuclear neutrophils (PMN) (Levy and Ruebner, 1967), another potential source of ROI and depletion of this cell type protects liver against endotoxaemia (Hewett et al., 1992). A non-oxygen-derived radical such as nitric oxide (NO), released by LPS-activated Kupffer cells and hepatocytes (Billiar et al., 1989a, 1989b; Nussler et al., 1992), can also react with O_2^- *in vitro* to form the peroxynitrite anion which can oxidize sulphydryl groups and generate the highly reactive $OH^{\bullet}$ during spontaneous decomposition (Beckman et al., 1990; Radi et al., 1991).

ROI may possibly mediate activation of the nuclear factor kB (NF-kB), a transcription factor that activates a multitude of genes encoding signalling and defence proteins (Schreck et al., 1992a). These include the acute-phase proteins which are believed to protect against cell damage and which are induced by LPS and cytokines or by trauma or inflammation (Koj, 1985).

LPS is known to induce HO activity of liver and Kupffer cells *in vivo* and of erythrophagocytic macrophages *in vitro* (Gemsa et al., 1974). We have shown that LPS and cytokines like interleukin-1 (IL-1) and tumour necrosis factor (TNF) induce hepatic activity and mRNA of HO-1 and that IL-1 transcriptionally activates the hepatic HO-1 gene; a computer search enabled us to identify an NF-kB sequence in the promoter region of the rat HO-1 gene (Cantoni et al., 1991; Rizzardini et al., 1993).

We have now examined the role of thiols alone and after LPS in hepatic HO-1 mRNA induction *in vivo*. We reduced the hepatic glutathione content by giving BSO (Drew and Miners, 1984) or sustained its synthesis by using pharmacological agents like *N*-acetylcysteine (NAC) or *S*-adenosylmethionine (SAM) (Burgunder et al., 1989; Friedel et al., 1989). NAC might also act as a direct scavenging agent (Aruoma et al., 1989).

We present evidence that one of the factors regulating HO-1 mRNA accumulation in the liver is its glutathione content and that ROI are likely mediators of LPS-induced HO-1 mRNA accumulation. These findings are compatible with the suggestion that HO-1 might have protective functions in the liver too when defence mechanisms against oxidants are challenged.

MATERIALS AND METHODS

Animals and treatments

Adult male CD1 mice (Charles River Italy, Calco, Como, Italy) weighing 22–25 g were used. They were fasted overnight before treatment. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national and international laws and policies (EEC Council Directive 86/609, OJ L 358, 1, Dec. 12, 1987; NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985).

LPS (Westphal preparation from *Escherichia coli* 055:B5, Sigma, St. Louis, MO, U.S.A.) was given intraperitoneally (i.p.) in 0.2 ml of sterile, pyrogen-free saline (0.9% NaCl) at the indicated dose. BSO (Sigma) was dissolved in sterile, pyrogen-free saline with the aid of 0.1 M NaOH (final pH 7.5–8) and injected i.p.; 1,4-butanedisulphonic salt of SAM was kindly provided by BioResearch (Liscate, Milan, Italy). NAC (kindly provided by Zambon, Bresso, Milan, Italy) was dissolved in water with the aid of 0.1 M NaOH (final pH 4.5–5) and was given by gavage 45 min before LPS at a dose of 1 g/kg. Controls received the same volumes of saline i.p. and/or water orally.

The animals were killed under ether anaesthesia at the indicated times, livers were quickly removed, rinsed with sterile saline, frozen in liquid nitrogen and stored at −80 °C.

Biochemical parameters

Glutathione content of livers was determined as total glutathione (glutathione), reduced glutathione (GSH) or oxidized glutathione (GSSG) according to the method of Griffith (1980).

Northern-blot analysis

Northern-blot analysis was performed according to standard procedures (Sambrook et al., 1989). Total RNA was extracted from approximately 0.5 g of liver according to the guanidinium isothiocyanate/CsCl method of Chirgwin et al. (1979). Total RNA (20 μg) was denatured, electrophoresed on 1.2% agarose/6% formaldehyde gels and transferred to synthetic nylon membranes (Gene Screen Plus: New England Nuclear, Boston, MA, U.S.A.) by capillary blotting; RNA transfer to membranes was checked by u.v. irradiation as shown in each Figure. These membranes were hybridized with the ^{32}P-labelled *Eco*RI/*Hin*d III restriction fragment (nucleotide residues 88–971) excised from the rat haem oxygenase cDNA pRHO1 obtained from Dr. S. Shibahara (Shibahara et al., 1985, 1987). The DNA fragment was labelled with α-[^{32}P]dCTP by the random priming method (Feinberg and Vogelstein, 1983). Hybridization was performed at 60 °C overnight in a solution containing 1 M NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulphate (Sigma), 100 μg of salmon sperm DNA/ml (Boehringer, Mannheim, Germany) and (1–2) $\times 10^6$ c.p.m. of labelled probe/ml. The membranes were washed twice with 2 × SSC/1% SDS (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 30 min at 65 °C and with 0.1 × SSC for 30 min at room temperature. The membranes were dried and exposed to Kodak X-Omat X-ray films with two intensifying screens at −80 °C for autoradiography. Autoradiograms were analysed densitometrically with the IBAS 2.0 (Kontron-Zeiss, PC 286) image analysis system.

RESULTS

Effect of increasing doses of BSO on liver glutathione concentrations and HO-1 mRNA levels

BSO is a selective inhibitor of glutathione biosynthesis which lowers the tissue glutathione concentration by specifically acting on γ-glutamylcysteine synthetase (γ-GCS). Table 1 shows the changes in glutathione content in livers of mice 4 h after increasing i.p. doses of BSO; at this time-point the effect of BSO on total hepatic glutathione is maximum according to Drew and Miners (1984). Glutathione was reduced to about 50% of control content by 0.6 g/kg BSO and depletion was as high as 80% with the largest dose (1.6 g/kg).

In the livers of mice given this high dose (1.6 g/kg), we measured GSSG (0.04 ± 0.01 and 0.09 ± 0.01 mg/g of liver in the control and BSO groups respectively) and GSH (1.10 ± 0.06 and 0.27 ± 0.07 mg/g of liver respectively). Thus, besides the massive

Table 1 Effect of increasing doses of BSO on hepatic glutathione content

Each value is the mean ± S.E.M. of at least three animals. Mice were killed 4 h after i.p. BSO. In parentheses, percentages of the saline group. Statistical significance was evaluated by Duncan's test.

Treatment	Glutathione (mg/g of liver)
Saline	1.49 ± 0.07
BSO 0.15 g/kg	1.00 ± 0.07 (67)*
BSO 0.60 g/kg	0.74 ± 0.05 (49)*†
BSO 1.20 g/kg	0.34 ± 0.05 (23)*‡§
BSO 1.60 g/kg	0.28 ± 0.07 (19)*‡§

*$P < 0.01$ versus saline group.
†$P < 0.05$ versus 0.15 g/kg BSO group.
‡$P < 0.01$ versus 0.15 g/kg BSO group.
§$P < 0.01$ versus 0.6 g/kg BSO group.

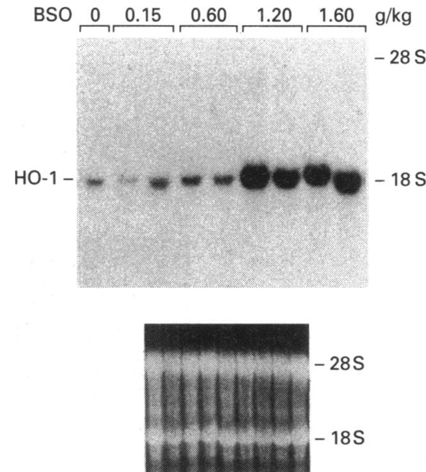

Figure 1 Induction of HO-1 mRNA by BSO in mouse liver

Total RNA (20 μg for each lane) was extracted for Northern-blot analysis from livers of mice killed 4 h after i.p. saline (0.2 ml) or increasing doses of BSO (0.15–1.6 g/kg). The position of the size markers (28 S and 18 S rRNA) is indicated. The lower part of the Figure shows the ethidium bromide-stained membrane, indicating that approximately equal amounts of RNA were added in each lane.

depletion of GSH, in the BSO group GSSG was more than doubled, reaching 26 % of the total glutathione in comparison to 4 % in the control group ($P < 0.05$ by Student's t test). However in terms of absolute amounts the levels of GSSG were quite low in both control and BSO livers in comparison to GSH.

In the same livers we measured the levels of HO-1 mRNA by Northern-blot analysis of total hepatic RNA using rat cDNA as a probe (Shibahara et al., 1985; 1987). As shown in Figure 1, control and BSO-treated livers showed a single mRNA band with mobility equivalent to that of the 18 S ribosomal RNA. The size of the detected mRNA is consistent with that expected from the rat and human HO-1 transcript (approximately 1800 nucleotides long for the rat mRNA and 1700 for the human counterpart) (Shibahara et al., 1985; Yoshida et al., 1988).

BSO increased accumulation of HO-1 mRNA, this effect being greater as the depletion of glutathione and the dose of BSO increased. Thus 50 % glutathione depletion (0.6 g/kg BSO) resulted in a small increase of transcript, about 2-fold, while maximal accumulation (about 20-fold) coincided with about 80 % glutathione depletion (1.2 g/kg and 1.6 g/kg BSO).

Effect of glutathione depletion with BSO on the accumulation of hepatic HO-1 mRNA after LPS

We investigated whether there was a relationship between the levels of glutathione and the accumulation of HO-1 mRNA after LPS. We lowered the liver glutathione content with two doses of BSO (one low and one high), and gave LPS 4 h after BSO.

In the first experiment we pretreated mice with a low BSO dose (0.3 g/kg, i.p.) that depleted hepatic glutathione and GSH by 40 % without significantly changing hepatic HO-1 mRNA (Table 2 and Figure 2a).

The low dose of LPS (0.1 mg/kg i.p.) we employed caused marked accumulation of HO-1 mRNA as early as 1 h after administration (Figure 2a). Glutathione and GSH were not

Table 2 Effect of LPS on hepatic glutathione, GSH, GSSG and HO-1 mRNA in control and BSO-treated mice

BSO (0.3 g/kg, i.p.) was injected 4 h before LPS (0.1 mg/kg, i.p.). Control mice received the corresponding vehicles (saline, 0.4 ml and 0.2 ml i.p.) and each treated group received the vehicle of the drug not administered. Mice were killed 1 h after LPS. Data are mean ± S.E.M. of at least four mice per group.

	Glutathione (mg/g of liver)	GSH* (mg/g of liver)	GSSG (mg/g of liver)	Relative HO-1 mRNA level†
Control	1.73 ± 0.12	1.67 ± 0.11	0.06 ± 0.01 (3.3)‡	1
LPS (0.1 mg/kg)	1.53 ± 0.08	1.47 ± 0.09	0.06 ± 0.02 (3.7)	27
BSO (0.3 g/kg)	1.09 ± 0.13§‖	1.02 ± 0.12§‖	0.07 ± 0.01 (6.2)	2
BSO + LPS	0.77 ± 0.11§¶	0.74 ± 0.11§¶	0.03 ± 0.01 (3.9)	217**

* GSH was obtained from the difference between the measurements of glutathione and GSSG (Griffith, 1980).
† Values are the relative HO-1 mRNA levels after densitometric analysis of the autoradiogram of Figure 2a.
‡ In parentheses, percentages of the respective glutathione value.
§ $P < 0.01$ in comparison to saline group by Duncan's test.
‖ $P < 0.05$ in comparison to LPS group by Duncan's test.
¶ $P < 0.01$ in comparison to LPS group by Duncan's test.
**$P < 0.01$ in comparison to the sum of the values of LPS and BSO separately by Tukey–Kramer's test.

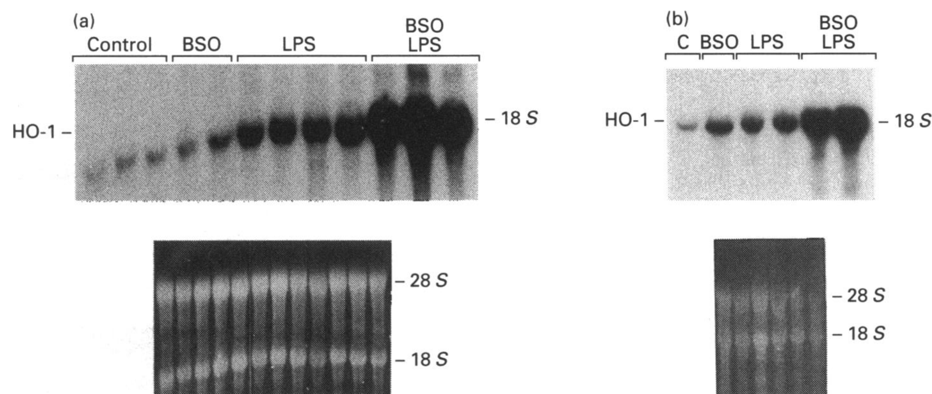

Figure 2 Syngergistic effect between BSO and LPS in the induction of HO-1 mRNA in mouse liver

(**a**) and (**b**) The Northern-blot analysis of total RNA (20 μg for each lane) extracted from livers of mice treated with BSO or LPS or both. BSO was injected i.p. at doses of 0.3 g/kg (**a**) or 1.6 g/kg (**b**) 4 h before LPS (0.1 mg/kg i.p.). Control mice received the corresponding vehicles (saline, 0.4 ml and 0.2 ml i.p.) and each treated group received the vehicle of the drug not administered. Mice were killed 1 h (**a**) or 2 h (**b**) after treatment with LPS. The position of the size marker (18 S rRNA) is indicated. The lower parts of the panels are as described in Figure 1.

changed in this group of animals, both being 88 % of the control value (Table 2).

The low BSO dose had a strong synergistic effect on LPS-induced accumulation of HO-1 mRNA; densitometric quantification of the autoradiogram in Figure 2a indicated that the induction of HO-1 mRNA rose from 27 times the control level after LPS to 217 times after LPS and BSO (Table 2).

The group receiving BSO and LPS had also the lowest glutathione and GSH content (both 44 % of the control value) (Table 2); these were lower than in the BSO group (63 % and 61 % of control), but the differences were not significant. With this low BSO dose, GSSG did not differ in these groups when comparing absolute amounts or percentages with the respective glutathione content (Table 2).

In the second experiment we pretreated mice with a high BSO dose (1.6 mg/kg, i.p.) that as expected (Table 1) depleted hepatic glutathione by 80 % (data not shown). This extensive depletion increased HO-1 mRNA transcript itself by about 15-fold, but, in the combined treatment, induction of HO-1 mRNA content by LPS was again potentiated. In fact the concomitant glutathione depletion and LPS treatment raised HO-1 mRNA by about 60-fold in comparison to a 15-fold increase in the LPS group (Figure 2b).

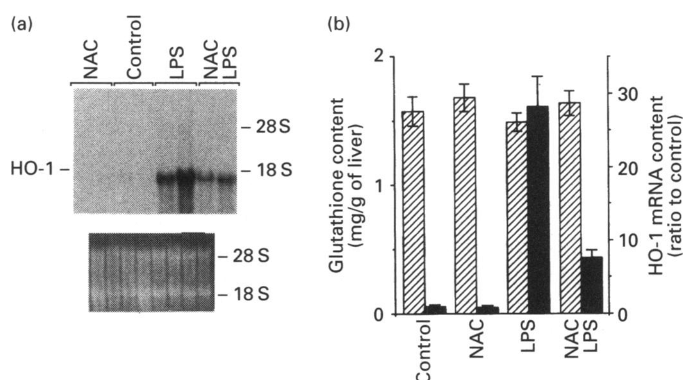

Figure 3 Effect of NAC pretreatment on LPS-induced HO-1 mRNA accumulation in mouse liver

(**a**) The Northern-blot analysis of total RNA (20 μg for each lane) extracted from livers of control mice and of mice treated with NAC, LPS or both. NAC was administered orally (1 g/kg) 45 min before LPS (0.1 mg/kg, i.p.). Control mice received the corresponding vehicles (water, 0.2 ml, orally and saline 0.2 ml, i.p.) and each treated group received the vehicle of the drug not administered. Mice were killed 1 h after LPS. The position of the size markers (28 S and 18 S rRNA) is indicated. The lower part of the panel is as described in Figure 1. (**b**) The hepatic glutathione values $\pm$ S.E.M. (▨) and the HO-1 mRNA contents $\pm$ S.E.M. (■) from two experiments are shown.

Effect of NAC and SAM on HO-1 mRNA accumulation and glutathione level in normal and glutathione-depleted mice treated with LPS

Compounds like NAC or SAM that are antioxidants and/or precursors of glutathione were tested for their capacity to counteract the LPS-induced HO-1 mRNA accumulation. Figure 3a shows the Northern-blot analysis of a representative experiment with NAC: 1 g/kg, orally, 45 min before LPS, reduced the HO-1 mRNA accumulation induced by LPS (0.1 mg/kg). Figure 3b (full bars) shows the densitometric quantification of the autoradiograms of two experiments. LPS caused marked accumulation of HO-1 mRNA (28 times the control), but NAC pretreatment limited this induction to one quarter. Figure 3b (hatched bars) shows the glutathione measurements from two experiments. Glutathione content was the same in all groups, though the group receiving LPS alone showed a tendency to a lower glutathione level than the controls, while NAC seemed to keep glutathione higher. In all groups GSSG was about 3–5 % of glutathione content (data not reported).

Pretreatment with SAM (1 g/kg, i.p., 45 min before LPS) did not modify LPS induction of HO-1 mRNA accumulation (data not reported) even though glutathione content was significantly raised ($P < 0.05$, by Duncan's test) in the SAM and SAM plus LPS group (1.94 $\pm$ 0.09 mg/g of liver and 1.86 $\pm$ 0.071 mg/g of liver respectively) in comparison to the LPS group (1.57 $\pm$ 0.04 mg/g of liver). The control value was 1.800 $\pm$ 0.13 mg of glutathione/g of liver.

We then tested the effect of NAC in extensively glutathione-depleted mice (Figure 4). Treatments are described in Figure 4a. BSO at the high dose (1.6 g/kg) again depleted the hepatic glutathione content by about 80 % (controls 1.50 $\pm$ 0.03 mg/g of liver) and NAC did not restore it (Figure 4c, hatched bars), as

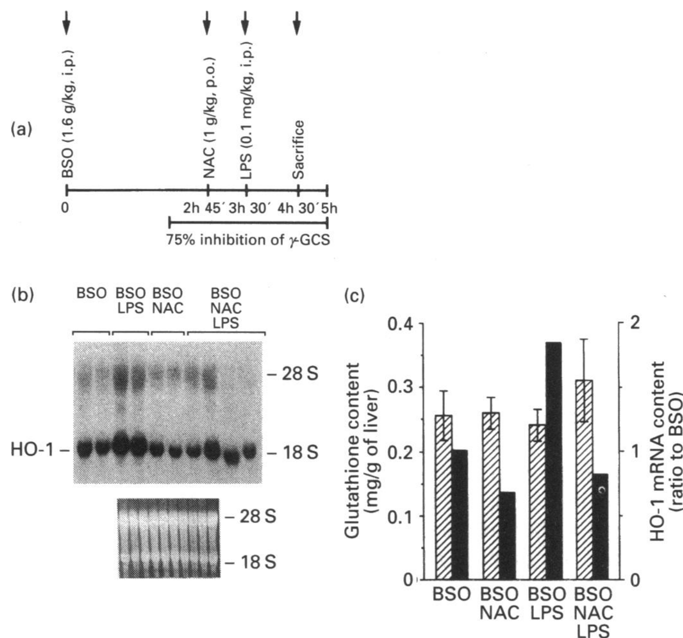

Figure 4 Effect of NAC on LPS-induced hepatic HO-1 mRNA accumulation in GSH-depleted mice

(**a**) The treatment schedule of the experiment. BSO (1.6 g/kg, i.p.) was given to all groups at time 0; NAC (1 g/kg, orally) and/or LPS (0.1 mg/kg, i.p.) were then given at the times indicated. At the same times the BSO group received the vehicles of the drugs administered to the other groups, i.e. water (0.2 ml, orally) and saline (0.2 ml, i.p.) and every group received the vehicle of the drug not administered. (**b**) The Northern-blot analysis of total RNA (20 μg for each lane) extracted from livers of mice treated following the scheme in (**a**). The position of the size markers (28 S and 18 S rRNA) is indicated. The lower part of the panel is as described in Figure 1. (**c**) The hepatic glutathione content of at least three animals per group (▨) and the ratio between the HO-1 mRNA content of each group and that of the BSO group after densitometric analysis of the autoradiogram of (**b**) (■).

was to be expected since when it was administered glutathione synthesis was at least 75% blocked by BSO (Drew and Miners, 1984). NAC counteracted the LPS-induced increase in HO-1 mRNA accumulation in BSO-pretreated mice too, so that induction was comparable in groups treated with BSO alone or with the combination of BSO, NAC and LPS and was less than half of that elicited by the combination of BSO and LPS (Figure 4b). In the absence of LPS, NAC slightly lowered HO-1 mRNA induction in glutathione-depleted mice. Figure 4c (full bars) shows the HO-1 mRNA levels after densitometric analysis of the autoradiogram of Figure 4b, taking the value of the BSO group as one. However in the BSO group, HO-1 mRNA was already induced over the control (27-fold) (data not shown), as in previous experiments (Figure 1 and Figure 2b).

DISCUSSION

Eukaryotic cells continuously produce ROI as side-products of electron transfer reactions (Halliwell and Gutteridge, 1989) and the intracellular concentrations of these intermediates seem to be finely tuned by specific mechanisms. One of these mechanisms is the intracellular thiol level that constitutively protects cells against oxidative damage. BSO is an inhibitor of glutathione synthesis that does not affect cytochrome *P*-450 or conjugating enzyme activity (Drew and Miners, 1984). Thus it is preferable to other glutathione-depleting agents as a specific depletor of tissue glutathione in investigations of molecular mechanisms.

The findings presented in this paper indicate, to our knowledge for the first time, the induction of HO-1 transcript in the liver of the intact mouse following glutathione depletion with BSO. The inductive response depends on the level of depletion: up to 50% depletion, HO-1 mRNA accumulation is slightly affected, whereas maximum depletion (about 80%) coincides with maximum induction. The differences in mRNA accumulation between normal and glutathione-depleted mice might be due to the inductive response being influenced by the accumulation of increased levels of ROI produced by basal cellular metabolic activity or by activation of enzymic systems producing these intermediates, like xanthine oxidase (Halliwell and Gutteridge, 1989; Cighetti et al., 1993). In fact the generation of active oxygen intermediates in cells through exposure to u.v. A or H_2O_2 can enhance HO-1 synthesis and transcriptionally activate the HO-1 gene (Keyse and Tyrrell, 1989a, 1989b; Keyse et al., 1990). Glutathione depletion might affect other cellular functions involved in the regulation of gene expression, like redox mechanisms that mediate protein factor binding to DNA, activation of transcriptional factors and membrane integrity, whose alterations might affect signalling mechanisms (Abate et al., 1990; Staal et al., 1990; Lotscher et al., 1979). Our results are in agreement with reports that under conditions of glutathione depletion by BSO, HO-1 mRNA is induced in the newborn rat brain (Ewing and Maines, 1993) and in human fibroblasts (Lautier et al., 1992). Additionally, depletion of glutathione by compounds acting through different mechanisms induced HO-1 (Oguro et al., 1990; Saunders et al., 1991).

Induction of HO *in vivo* in conditions of oxidant stress may have a protective function, since it results in increased formation of bilirubin, a physiological antioxidant (Stocker et al., 1987), and removes haem, which is a reactive form of iron that can participate in oxygen-radical reactions (Gutteridge and Smith, 1988). An additional role for HO-1 induction in conditions of GSH depletion might be regulation of cyclic GMP production by guanylate cyclase. In fact depletion of glutathione inhibits NO synthase activity (Ghigo et al., 1993) and in the brain it was suggested that CO generated by induced HO-1 activity in conditions of glutathione depletion could function in the same capacity as NO (Maines et al., 1993). HO-1 might play a comparable role in the liver.

We have previously shown that HO-1 mRNA and enzymic activity are very sensitive to LPS and cytokines, but the mechanism(s) of action are not yet known. There is evidence that both ROI and nitrogen intermediates are involved in the mechanism(s) of LPS-mediated hepatotoxicity (Ghezzi et al., 1993; Shedlofsky and McClain, 1991).

Reactive intermediates are released by Kupffer cells and infiltrating PMN activated by LPS (Bautista et al., 1990) or by induced enzymic systems like xanthine oxidase (Ghezzi et al., 1984) or NO synthase(s) (Nussler et al., 1992). Endotoxaemia lowered GSH in rat and mouse liver (Kunimoto et al., 1987; Sugimoto et al., 1991) and cell lines *in vitro* released more O_2^- and H_2O_2 into the medium when triggered with small amounts of LPS (Schreck et al., 1992b).

We used a low dose of LPS to avoid general toxicity. In our experiments we constantly observed a small reduction in the amount of glutathione in LPS-treated mice, although it did not reach statistical significance probably because it was measured concomitantly with HO-1 mRNA, i.e. very shortly after administration of LPS.

We used NAC as antioxidant and GSH depletion by BSO to investigate whether reactive intermediates were involved in HO-1 mRNA induction by LPS. Other investigators have used NAC to protect against LPS toxicity (Bernard et al., 1984; Peristeris et

al., 1992), while GSH depletion by phorone (di-isopropylidene acetone) enhanced the LPS-induced release of hepatic N^1-acetylspermidine, a suggested indicator of the radical-producing potency of several drugs (Sugimoto et al., 1991). Our results support the suggestion that reactive intermediates mediate HO-1 mRNA induction by LPS.

NAC is a non-toxic drug that enters cells readily and serves either as a precursor for glutathione, replenishing the intracellular pool of cysteine, or as a scavenger. NAC inactivates hypochlorous acid (HOCl) and hydroxyl radical (OH$^\cdot$) while minimally scavenging superoxide anion and H_2O_2 *in vitro* (Aruoma et al., 1989).

NAC pretreatment resulted in a marked decrease of HO-1 transcript induction by LPS, even when glutathione synthesis was blocked by BSO. Thus under our conditions it is likely that NAC acts as a direct scavenger of some specific reactive intermediate and/or counteracts some specific LPS-induced oxidative reaction that triggers HO-1 mRNA induction. Alternatively, NAC might affect some of the modifications, induced by LPS, that sustain increased formation of reactive intermediates. In the lungs NAC appears to reduce the infiltration of PMN induced by IL-1, a cytokine released after LPS (Leff et al., 1993). However in both cases the result would be a reduction in the amount of reactive intermediates. In this function glutathione cannot replace NAC since SAM, although it significantly raised glutathione levels, did not affect HO-1 mRNA induction.

Lowering liver glutathione content with BSO markedly affected the extent of HO-1 mRNA induction after LPS. Comparable evidence was obtained with human fibroblasts, in which glutathione depletion with BSO strongly increased the induction of HO-1 mRNA in u.v. A- or H_2O_2-treated cells (Lautier et al., 1992). With the BSO dose that extensively depleted glutathione, the effect on HO-1 mRNA induction was probably limited by overall toxicity. Greater sensitivity to the cytotoxic action of u.v. A radiation was reported after extensive depletion of glutathione with BSO in human skin fibroblasts, and concomitantly potentiation of HO-1 mRNA induction declined (Lautier et al., 1992).

Our interpretation is that the lowered glutathione level enhanced the inducible response of HO-1 by allowing higher levels of radical intermediates derived from both LPS-induced and normal metabolic activities to persist in the cell. A further contribution to the synergism might come from the defective actions of mechanisms through which glutathione may control HO-1 gene expression, as discussed earlier with reference to the experiments with BSO alone.

Another indication that HO-1 induction by LPS is very likely sustained by the formation of reactive intermediates comes from our preliminary experiments (data not shown) in which induction of HO activity by LPS was in fact increased after pretreating mice with L-NAME, an inhibitor of NO synthase (Rees et al., 1990). NO protects against cytotoxicity from ROI and inhibits PMN superoxide anion production (Wink et al., 1993; Clancy et al., 1992). Inhibition of NO-synthases during endotoxaemia was shown to be deleterious (Billiar et al., 1990; Wright et al., 1992).

LPS activates the transcription factor NF-kB *in vitro* and *in vivo* (Schreck et al., 1992a; Freedman et al., 1992) and *in vitro* this activation was blocked by the antioxidant pyrrolidine dithiocarbamate (Schreck et al., 1992b). NF-kB is believed to be primarily an oxidative stress-responsive transcription factor that activates a variety of genes mainly involved in inflammatory, immune and acute-phase responses. This pathway is shared by a wide variety of agents including some that also affect HO-1, like phorbol esters, inflammatory cytokines, u.v. light and H_2O_2. ROI might serve as a common messenger in the activation of NF-kB (Schreck et al., 1991).

Intracellular thiols play a key role in regulating NF-kB activation *in vitro* and it was suggested that they could act at one or more points in the signal transduction pathway (Staal et al., 1990). HO-1 gene expression is regulated at the transcriptional level by agents causing oxidative stress, thiol group reactive agents or cytokines (Keyse et al., 1990; Rizzardini et al., 1993). Recognition sequences for NF-kB were identified in the promoter region of rat and human HO-1 genes (Rizzardini et al., 1993; Lavrovsky et al., 1993). Therefore modulation of the HO-1 mRNA inducible response by changes in the glutathione status and/or LPS treatment is consistent with the theory that signalling mechanism(s) involving NF-kB might be important in regulating HO-1 gene expression.

On HO-1 genes we have previously identified two IL-6 responsive elements and sequences for NF-IL6, a nuclear factor involved in the hepatic response to inflammation (Rizzardini et al., 1993). Other authors have suggested that induction of HO-1 expression by glutathione depletion might be due to activation of the antioxidant response element, whose sequence appears to be present in the HO-1 regulatory region (Maines et al., 1993). The presence of all these different regulatory sequences highlights the existence of a complex upstream regulatory region which should make HO-1 highly sensitive to regulation at the transcriptional level in conditions of oxidative stress.

In conditions of oxidant damage in vascular endothelium HO induction was associated with induction of the iron-binding protein ferritin which would behave as the ultimate cytoprotectant (Balla et al., 1992); furthermore oxidative stress resulting from u.v. A irradiation of human skin fibroblasts led to a HO-dependent increase of ferritin (Vile and Tyrrell, 1993). It would be interesting to test this in conditions of glutathione depletion or after LPS which does in fact lower the circulating iron level shortly after administration (Klasing, 1984) and induces iron accumulation in the liver (Van Snick et al., 1974). In a model of inflammation with turpentine, ferritin synthesis in the liver was increased, preceding a decrease in plasma iron (Konijn and Hershko, 1977).

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