Cytokine induction of haem oxygenase mRNA in mouse liver

Interleukin 1 transcriptionally activates the haem oxygenase gene

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Accumulation of the mRNA coding for haem oxygenase (HO, EC 1.14.99.3) was stimulated by treating mice with endotoxin (lipopolysaccharide, LPS; 20 μg/mouse intraperitoneally), suggesting that haem catabolism is a target of infection and inflammation *in vivo*. Therefore various cytokines, possible mediators for the biological responses to LPS, were administered intraperitoneally to mice, and the levels of HO mRNA were measured by Northern-blotting analysis using the rat HO cDNA as a probe [Shibahara, Müller, Taguchi and Yoshida (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 7865–7869]. Marked induction of HO mRNA was observed 2 h after administration of interleukin 1 (IL-1) (34-fold) and tumour necrosis factor (19.5-fold) (5 μg/mouse), whereas interleukin 6 (6.2 μg/mouse) was much less active (3.5-fold) and interleukin 2 (25 μg/mouse) and interferon-γ (3 μg/mouse) were ineffective. HO mRNA induced by the cytokines or LPS accumulated rapidly (maximum at 1–2 h after administration), preceding the elevation of HO enzymic activity. Treatment of mice with IL-1 stimulated the transcription of the HO gene by 4-fold, as assessed by *in vitro* nuclear-run-on assay. These results indicate that enzymic haem catabolism in the liver is a process inducible *in vivo* by inflammatory cytokines, which up-regulate HO synthesis at the transcriptional level. Increased removal of haem might be part of the protective mechanisms elicited by the acute-phase response, possibly to reduce the pro-oxidant state of the cell.

INTRODUCTION

Microsomal haem oxygenase (HO, EC 1.14.99.3) catalyses the initial and rate-limiting step in haem catabolism, the oxidative degradation of haem to biliverdin. The haem molecule (a complex of ferrous iron and protoporphyrin IX) has a central role in life in eukaryotes, since it is the prosthetic moiety of haemoproteins involved in cell respiration, energy generation and oxidative biotransformation. The regulation of HO activity is thus fundamental for the homoeostasis of the cell [1]. In experimental animals, in conditions such as spontaneous or chemically induced liver tumours [2] and Gram-positive bacterial infections [3], the activity of HO is significantly increased. HO activity is also elevated in whole animal tissues (particularly kidney and liver) by its natural substrate haem, and by heavy metals, xenobiotics, endocrine factors and synthetic metalloporphyrins [1]. Furthermore, HO is a heat-shock protein, being induced upon heat shock [4,5] and also a stress protein induced upon oxidative stresses [6].

One of the compounds inducing HO *in vivo* is endotoxin (lipopolysaccharide, LPS) [1], used as a model for the acute-phase response, i.e. the systemic reaction to tissue damage and inflammation caused by trauma, infections and other acute insults. Acute-phase response is characterized in the liver by the synthesis of a number of plasma proteins known as acute-phase reactants [7], and cytokines such as interleukins 1 and 6 (IL-1 and IL-6) and tumour necrosis factor (TNF) are presently considered the most important mediators of this effect [8,9]. IL-1 and IL-6 induced HO *in vitro* [10,11], and recently we found that IL-1 and TNF induce HO activity *in vivo* and that IL-1 might be mainly responsible for the induction of HO activity by LPS [12].

Our study further investigated the mechanism(s) underlying the induction of hepatic HO activity during the acute-phase response resulting from the *in vivo* administration of LPS and cytokines. Since the rat HO gene as well as its human counterpart have recently been isolated and are available [13,14], we examined the transcriptional regulation of HO gene using the rat HO cDNA as a probe. The results presented here demonstrate that the elevation of the HO enzymic activity by IL-1 and TNF, as well as by LPS, is preceded by rapid accumulation of the HO mRNA, which is explained partly by transcriptional activation of the gene itself and by other post-transcriptional events.

MATERIALS AND METHODS

Animals and treatments

Adult male CD1 mice, C3H/HeN and endotoxin-resistant C3H/HeJ mice (22–24 g) were obtained from Charles River, Calco, Como, Italy. LPS (Westphal preparation from *Escherichia coli* 055:B5) was from Sigma, St. Louis, MO, U.S.A. TNF (recombinant human TNF-α; specific activity 8.1×10^6 units/mg) was kindly given by BASF/Knoll, Ludwigshafen, Germany. IL-1 (recombinant human IL-1β) was kindly given by Sclavo, Siena, Italy. IL-6 (recombinant human IL-6) was kindly given by Serono. IL-2 (recombinant human IL-2) was kindly given by Glaxo. IFN (mouse rIFN-γ; specific activity 1×10^7 units/mg) was kindly given by Dr. G. R. Adolf, Ernst-Boehringer Institut, Vienna, Austria.

All agents were injected into experimental animals intraperitoneally in 0.2 ml of sterile, pyrogen-free saline (0.9 % NaCl). The treated animals were killed at the indicated time and the liver was used for further analysis.

Abbreviations used: HO, haem oxygenase; LPS, lipopolysaccharide; IL-1, interleukin 1; TNF, tumour necrosis factor; IL-6, interleukin 6; IL-2, interleukin 2; IFN, interferon-γ; NF-kB, nuclear factor kB (nuclear factor that binds the immunoglobulin k light chain gene enhancer); NF-IL6, nuclear factor that binds to an IL-1-responsive element in the IL-6 gene.

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HO activity

HO activity was measured on a post-mitochondrial supernatant as previously described [12].

Northern-blotting analysis

Northern-blotting analysis was performed according to standard procedures [15]. Total RNA was extracted from approx. 0.5 g of liver according to the guanidinium isothiocyanate/CsCl method of Chirgwin et al. [16]. Total RNA (20 μg) was denatured, electrophoresed on 1.2 %-agarose/6 %-formaldehyde gels, transferred to synthetic nylon membranes (GeneScreen Plus; New England Nuclear, Boston, MA, U.S.A.) by capillary blotting and hybridized with the ^{32}P-labelled *Eco*RI–*Hin*dIII restriction fragment (nucleotide residues 88–971) excised from the rat haem oxygenase cDNA pRHO1 obtained from Dr. S. Shibahara [4,17]. The DNA fragment was labelled with α-[^{32}P]dCTP by the random priming method [18]. 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 $\times$ SSC/1 % SDS (1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) for 30 min at 65 °C and with 0.1 $\times$ 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 using a digitized image analyser (RAS 3000; Loats System, Amersham). Signals generated from the HO probe hybridization were normalized to those produced by rehybridization of the same filter with a mouse actin cDNA probe [19].

Nuclear transcription run-on assay

Nuclei were isolated from mouse liver 1 h after administration of saline (0.2 ml/mouse) or IL-1 (5 μg/mouse) by the method described by Marzluff and Huang [20]. The transcription elongation assay was performed by the method described by Greenberg and Ziff [21] with minor modifications. Briefly, approx. 1.2 $\times$ 10^7 nuclei were thawed on ice, pelleted and resuspended in 60 μl of glycerol buffer [40 % (w/v) glycerol/5 mM $MgCl_2$/0.1 mM EDTA/50 mM Tris, pH 8.3]. To the nuclear suspension, 20 μl of run-on buffer [25 mM Tris (pH 7.5)/12.5 mM $MgCl_2$/750 mM KCl], 5 μl of NTP mix (5 mM each of ATP, CTP and GTP), 1 μl of RNAase inhibitor (human placenta; Gibco, BRL) and 200 μCi/20 μl of [^{32}P]UTP (3000 Ci/mmol, Amersham) were added and the mixture was incubated at 30 °C for 30 min. The reaction was stopped by addition of 50 units of DNAase I (pancreatic deoxyribonuclease, RNAase-free, Amersham). After 1 min incubation at 30 °C, the mixture was deproteinized with 1.2 μg of proteinase K and the elongated transcripts were extracted with phenol/chloroform. Unincorporated isotope was eliminated by Sephadex G-50 spun-column chromatography [15]. Labelled elongated RNAs (minimum 2 $\times$ 10^6 c.p.m./ml) were hybridized to 10 μg each of the cDNA plasmids immobilized on nitrocellulose filters after denaturation by heat and alkali treatment. Prehybridization and hybridization were performed at 65 °C. The filters were washed in 0.2 $\times$ SSC/0.1 % SDS at 65 °C for 30 min and then in 0.2 $\times$ SSC in the presence of 1 μg/ml RNAase A at room temperature for 5 min. Autoradiography was performed as described above. The plasmid DNAs used for these experiments were rat HO cDNA [17], histone H2a cDNA [22], actin cDNA [19] and plasmid p106 without any insert as a negative hybridization control.

RESULTS

Levels of HO mRNA were measured by Northern-blotting analysis of liver RNA using rat HO cDNA as a probe [17]. As shown in Figure 1, treatment with LPS resulted in the accumulation of 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 rat and human HO transcripts (approx. 1800 nucleotide long for the rat mRNA and 1700 nucleotide long for the human counterpart) [17,23]. This increase was evident as early as 2 h after treatment; however, by

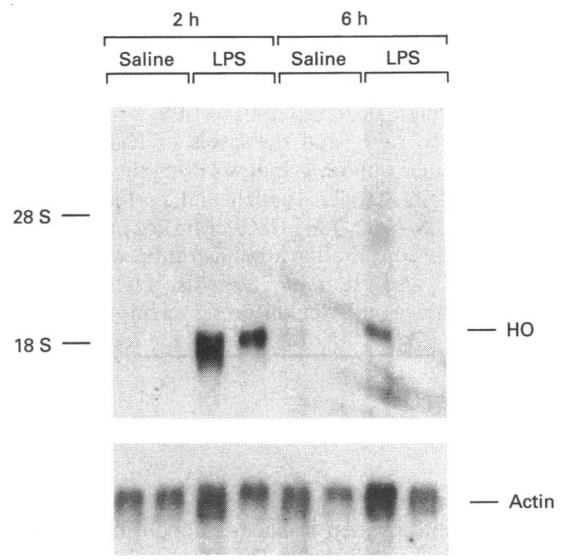

Figure 1 Induction of HO mRNA by LPS in mouse liver

Total RNA (20 μg for each lane) was extracted for Northern-blotting analysis from livers of mice, 2 or 6 h after treatment with saline or LPS (20 μg/mouse intraperitoneally). The positions of the size markers (28 S and 18 S rRNA) are indicated. The same filter was rehybridized with actin cDNA.

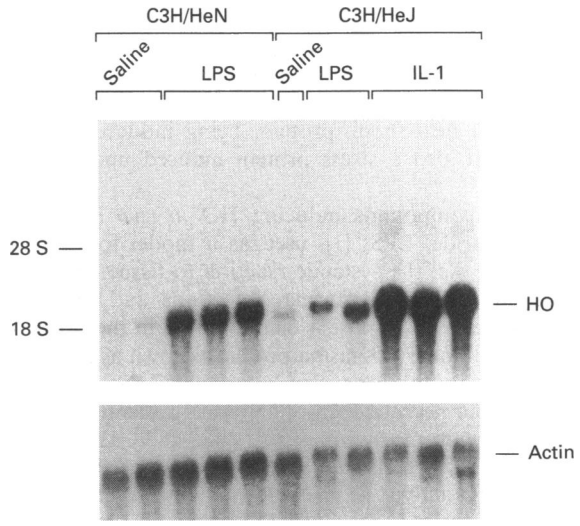

Figure 2 Induction of HO mRNA by LPS in normal and LPS-resistant mice

Total RNA (20 μg for each lane) was extracted for Northern-blotting analysis from livers of normal (C3H/HeN) and LPS-resistant (C3H/HeJ) mice, 2 h after treatment with saline or LPS (20 μg/mouse intraperitoneally). The last three lanes show HO mRNA from LPS-resistant mice treated with IL-1 (2.5 μg/mouse intraperitoneally). The positions of the size markers (28 S and 18 S rRNA) are indicated. The same filter was rehybridized with actin cDNA.

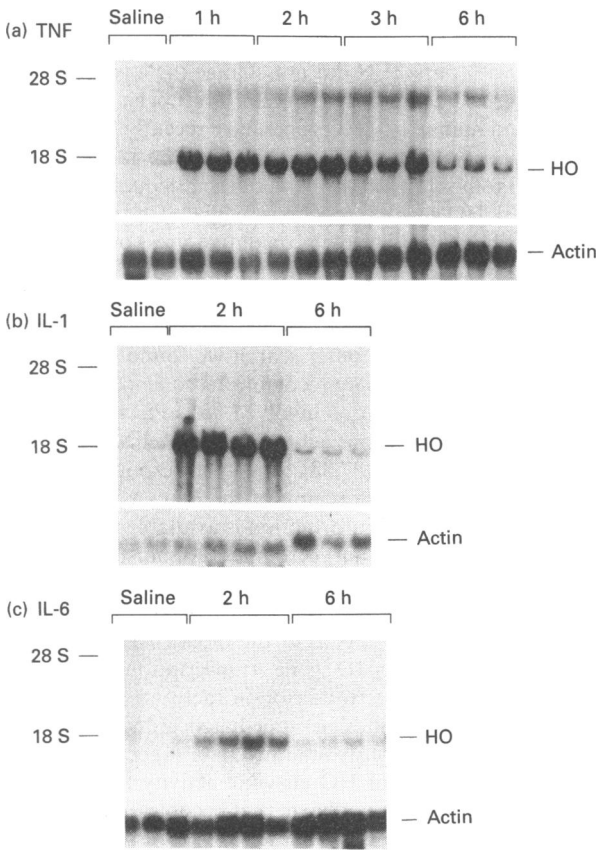

Figure 3 Kinetics of HO mRNA accumulation in mouse liver after cytokines

(**a**), (**b**) and (**c**) show the time course of hepatic mRNA accumulation after TNF (5 μg/mouse intraperitoneally), IL-1 (5 μg/mouse intraperitoneally) and IL-6 (6.2 μg/mouse intraperitoneally) respectively. Total RNA (20 μg for each lane) was isolated for Northern-blotting analysis at various times after treatment. The positions of the size markers (28 S and 18 S rRNA) are indicated. The same filters were rehybridized with actin cDNA.

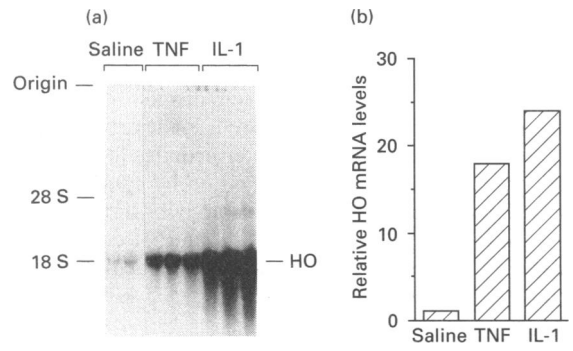

Figure 4 Comparison of the potency of TNF and IL-1 in inducing HO mRNA

(**a**) shows the Northern-blotting analysis of total RNA (20 μg for each lane) extracted from livers of mice treated with equimolar doses of TNF or IL-1 (5 μg/mouse intraperitoneally) and killed after 2 h. The position of the size markers (28 S and 18 S rRNA) is indicated. (**b**) shows the relative HO mRNA levels after densitometric analysis of the autoradiograms obtained from the same filter sequentially hybridized with HO cDNA and actin cDNA as control.

6 h, the amount of HO mRNA had decreased. The accumulation of HO mRNA preceded the elevation of HO enzymic activity, which had not increased by 2 h after treatment (5.6 ± 0.90 pmol/min per mg of protein in the control group versus

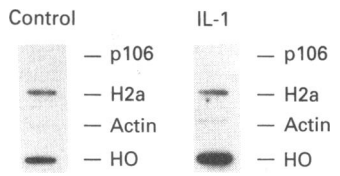

Figure 5 Effect of IL-1 on transcription of the HO gene in mouse liver

A nuclear run-on assay was performed on livers of mice treated with saline or IL-1 (5 μg/mouse intraperitoneally) Each sample was a pool of nuclei from three livers. The probes used are indicated. The plasmid p106 without insert was used as a negative hybridization control.

4.78 ± 0.59 pmol/min per mg of protein in the LPS group) and increased at 6 h (5.42 ± 0.5 pmol/min per mg of protein in the control group versus 10.2 ± 1.0 pmol/min per mg of protein in the LPS group; $P < 0.01$ by Student's t test).

To test whether hepatic HO mRNA induction is mediated by macrophage products, similar experiments were performed using the LPS-resistant strain. As shown in Figure 2, induction of HO mRNA by LPS (20 μg/mouse) at 2 h in C3H/HeJ, the LPS-non-responsive strain, was less than a quarter (2.5-fold) of the average induction (14-fold) obtained in the responsive C3H/HeN strain. Hepatic HO mRNA was increased in LPS-resistant C3H/HeJ mice receiving 2.5 μg of IL-1/mouse (30-fold) (Figure 2) or given serum obtained from normal C3H/HeN mice 90 min after treatment with LPS (results not shown).

Since some cytokines are known to mediate LPS activity, the levels of liver HO mRNA after injection of approximately equimolar amounts of TNF, IL-1 and IL-6 (respectively 5 μg/mouse for TNF and IL-1 and 6.2 μg/mouse for IL-6) were measured at different times. As shown in Figure 3(a), TNF treatment caused rapid and remarkable induction of the HO transcript, being already about 18-fold the control level 1 h after treatment, and approximately the same level of induction was maintained until 3 h (19.5-fold at 2 h and 16-fold at 3 h). The amount of the HO mRNA then decreased to 4-fold the control level at 6 h. Induction of enzymic activity temporally followed the increase in the mRNA level, being higher at 6 h than at 3 h (respectively 11.6 ± 0.8 pmol/min per mg of protein in the control group versus 19.4 ± 1.6 pmol/min per mg of protein and 24.8 ± 1.5 pmol/min per mg of protein respectively in the TNF/3 h and TNF/6 h groups; $P < 0.01$ by Student's t test at both times). Similarly to TNF, IL-1 (Figure 3b) produced a rapid and massive increase in hepatic HO mRNA by 2 h (34-fold); at 6 h, induction had almost completely declined (2-fold). Induction by IL-6 (Figure 3c) was about 3.5-fold after 2 h and it was no longer detectable at 6 h.

To compare the relative potency of the cytokines, hepatic RNA samples derived from mice 2 h after TNF or IL-1 injection (the time point at which maximum induction was observed in Figure 3) were loaded on the same gel and Northern-blotting analysis was performed. Densitometric quantification of the autoradiogram shown in Figure 4(a), normalized for the corresponding actin hybridization signals, demonstrated that the induction of HO mRNA by IL-1 and by TNF was 24-fold and 18-fold respectively (Figure 4b). IL-2 (25 μg/mouse) and IFN (3×10^4 units, i.e. 3 μg/mouse) did not appreciably increase hepatic HO mRNA levels 2 h after treatment (results not shown). To understand the mechanism of accumulation of 'steady-state' level of HO mRNA, a nuclear transcription run-on assay was performed after treatment with IL-1, the most potent cytokine so far tested. As shown in Figure 5, the amount of HO specific nuclear RNA was already detectable in basal conditions and

significantly increased upon induction by IL-1 (4-fold by densitometric analysis). The transcriptional activation of the HO gene is specific, since neither the level of the actin nor that of the histone H2a gene nuclear transcripts were changed under the same hybridization conditions.

DISCUSSION

We have recently shown that mouse hepatic HO enzymic activity is induced during the acute-phase response caused by LPS, IL-1 and TNF and that induction by LPS is very likely mediated by cytokines [12]. The present study provides evidence that induction of HO activity *in vivo* by LPS and cytokines is preceded by accumulation of its mRNA. Experiments with LPS-resistant C3H/HeJ mice confirmed that induction of HO mRNA after LPS was due mainly to mediator molecules rather than LPS itself, since LPS was not so effective as in normal mice. Since the mechanism of the LPS resistance of the C3H/HeJ mouse strain is thought to be a defective response of macrophages to LPS [24], it is likely that cytokines produced by macrophages are the responsible mediators. In fact, IL-1 was extremely potent in inducing hepatic HO mRNA in LPS-resistant mouse strain.

The cytokines IL-1, TNF and IL-6 are the main mediators of the acute-phase reaction [8,9,25–27] and they are part of a network in which each cytokine not only regulates the synthesis of the other cytokines, but also modifies their action at the target cell, either by synergism or by antagonism. IL-1 and TNF appear to act synergistically and induce their own synthesis and that of IL-6, while IL-6 does not induce its own synthesis nor that of IL-1 and TNF. On the contrary, IL-6 suppresses endotoxin and TNF-induced IL-1 production. Furthermore, IL-1 and TNF activate endothelial cells and induce proliferation of fibroblasts which are other known sources of cytokines, whereas IL-6 does not (for a review, see [25]). Therefore, it is very difficult to establish the role of each single cytokine *in vivo*, and even studies with different cell culture model systems do not fully clarify what might be the major regulator of acute-phase-protein synthesis as seen in inflammatory states in humans [28–30]. Helqvist et al. [10], in isolated rat islets of Langerhans, reported that IL-1 induced a 32 kDa protein suggested to be HO, and recently Mitani et al. [11] reported a slight increase of HO mRNA after treatment with IL-6 in the human hepatoma cell line Hep3B. *In vivo*, we observed that IL-1 and TNF, which share remarkable similarities in their biological properties, particularly the non-immunological ones, caused a massive increase in HO mRNA, while IL-6, which generally appears to be a weak inflammatory peptide, was much less active. This effect might be at least partly due to the differences outlined above between these three cytokines in their capacity to regulate the synthesis of other cytokines.

The increase in HO enzymic activity after TNF was less significant than that observed in mRNA level, and the same is likely after IL-1 [12]. This discrepancy might be explained by some post-transcriptional events, such as stability of the cytoplasmic mRNA and that of the active protein. It is noteworthy that there is again a discrepancy in the level of IL-1-mediated induction of the HO gene transcription assessed by nuclear run-on assay (4-fold) and that of the corresponding mRNA accumulation (34-fold). This could be interpreted as an indication that IL-1 not only activates the HO transcription, but also stabilizes the cytoplasmic mRNA, a mechanism that is suggested to be important for cytokine–cytokine synergy [31].

IL-2 and IFN had hardly any effect on hepatic HO mRNA 2 h after acute administration. Results for the role of IL-2 in induction of acute-phase proteins are lacking. IFN was reported to induce HO activity after 24 h; however, the dose used was more than 3 times higher than ours [32] and, at a dose comparable with ours, IFN did not change the levels of another well-characterized acute-phase protein, fibrinogen [33].

Rat [13] and human [14] HO genes have recently been isolated, and the similarity of the HO proteins between rat and human was reported to be 80% on the basis of their deduced amino acid sequences [23]. HO gene expression is regulated at transcriptional level by heat shock, heavy metals, oxidative stress or thiol-group-reactive reagents [4,34–36]. In fact, the 5′ flanking regions of the HO genes contain consensus sequences of potential regulatory significance that seem to be related to the stress response [13,12,32]. Through a computer search we found on HO genes sequences that perfectly share recognition sequences for NF-IL6 and NF-kB, nuclear factors involved in LPS and cytokine-mediated elevation of various acute-phase proteins in the liver in response to inflammation [37,38]. Two IL-6-responsive elements are present in the promoter region of the human HO gene [11].

Although no structural information is available for the mouse HO gene, our findings that cytokines administered to mice increase HO mRNA and transcriptionally activate the HO gene suggest that similar cytokine-responsive sequences might be present in the regulatory region of the mouse HO gene.

Haem also activates HO gene transcription [4], but the putative haem-responsive transcription factor(s) and the DNA sequence(s) involved in the haem-mediated induction have not yet been identified. Previously obtained data suggested that haem mediated the induction of HO enzymic activity after LPS [39], but the intracellular source of haem that becomes available is still questioned [1]. LPS, like IL-1 and IL-6, suppresses constitutive cytochrome *P*-450 gene expression [40,41], and this effect might create a temporary accumulation of haem. Further studies are required to clarify whether LPS, cytokines and haem share at least part of their molecular mechanism(s) of action on the HO gene.

Our results indicate that HO induction is part of the host's systemic reaction to cytokines. In this acute-phase response, levels of specific plasma and tissue proteins change, probably in order to protect against cell damage, although their specific function is not yet defined [7,42]. HO degrades haem, which is a reactive, low-molecular-mass form of iron able to participate in oxygen-radical reactions that can lead to the degradation of cellular macromolecules. In the mechanism(s) of LPS and cytokine-mediated hepatotoxicity, there is evidence of a role for both reactive oxygen and nitrogen intermediates [43].

In this context, transcriptional activation by cytokines of the HO gene and induction of HO enzymic activity should result in a transient reduction in the pro-oxidant state of cells, and this itself might be a defence mechanism, but elucidation of its specific functional role is still lacking.

We are grateful to Dr. S. Shibahara for his generous supply of rat HO cDNA and to P. Ghezzi for helpful discussions. This work was supported by the CNR (National Research Council, Rome, Italy), Contract no. 92. 01675, CT04 'Role of hepatic heme oxygenase (HO) in acute-phase response' and 'Progetto finalizzato Ingegneria genetics', Contract no. 91.00060. PF 99, and by the Associazione Italiana per la Ricerca control il Cancro (AIRC). F.F. is a recipient of a fellowship from the Fondazione 'Attilia Pofferi'.

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Received 13 July 1992/14 September 1992; accepted 24 September 1992