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Differential Induction of Heme Oxygenase in the Hepatocarcinoma Cell Line (Hep3B) by Environmental Agents

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Abstract In situ hybridization and Northern analysis of heme oxygenase (HO) mRNA was used to determine the induction and expression of HO by various environmental agents. Exposure of Hep3B cells to hemin (10 μM) for as little as 5 min resulted in significant production of HO transcripts and mRNA expression as seen by in situ hybridization. We followed the pattern of HO transcript accumulation by heme and results indicate that the peak of induction of HO by heme was reached between 10 and 20 minutes. Other metalloporphyrins were all effective in inducing HO mRNA after 1 h exposure. On the other hand, $CoCl_2$ caused accumulation of HO mRNA at a later time than seen with the metalloporphyrins. However, lipopolysaccharide (LPS) gave a more immediate effect on HO induction which was somewhat similar to heme in its time course. Direct measurements of HO activity revealed that enzyme activity could be detected after about 20 min exposure to hemin, and this activity was inhibited by tin protoporphyrin (SnPP). The different pattern of HO mRNA induction by LPS as contrasted with $CoCl_2$ suggests that LPS may act through a different translational factor, or stimulate free radical formation and the subsequent release of heme and induction of HO. These results indicate that heme causes accumulation of HO mRNA by a different mechanism than that of $CoCl_2$. Finally, LPS shares a concomitant effect on induction of HO as an acute phase reactant type protein.

Key words: heme, heme oxygenase, mRNA, environmental agents, metalloporphyrins, lipopolysaccharide, acute phase, Hep3B

Heme oxygenase [E.C.1.1499.3] (HO) is the rate-limiting enzyme for heme catabolism, and fluctuations in the activity of or expression of HO have pronounced effects on hematopoiesis and multiple cellular functions [Abraham et al., 1991a; Lutton et al., 1991]. It has been suggested that HO is one of the most important enzymes, and regulation of its expression may be critical for survival of the normal living process [Stocker, 1990; Mitani et al., 1991]. This becomes apparent when one realizes the ubiquitous role of heme in cellular functions, such as the cytochrome P450 metabolizing systems, cellular respiration, and the growth-differentiation process [Abraham et al., 1991a; Lutton et al., 1991].

Several classes of heat shock proteins have been described which are thought to protect or recover functions of various cellular proteins under stressed conditions [Schlesinger, 1990; Hershko, 1988]. Recently, it was reported that HO may function as a heat shock protein [Shibahara et al., 1987]. Keyse and Tyrrell [1989] suggested that HO may constitute part of the defense mechanism against oxidative damage produced by free radicals. In this respect, bile pigments produced by heme degradation can function as important antioxidants [Stocker, 1990]. Bacterial endotoxins such as lipopolysaccharides (LPS) are known to induce HO activity in rat liver, and it is clear that LPS can play a role in the inflammatory response and immune modulation [Bissel and Hammaker, 1976; Baumann et al., 1984]. Thus, it is possible that the induction of HO may be an essential event for some types of acute reactions and cellular protection.

We and others have shown that heme has beneficial effects on normal and disturbed hematopoiesis. However, excess heme causes a rapid increase in HO [Abraham et al., 1991a; Lutton et al., 1991]. Hepatic cells such as the Hep3B cell line have very active heme and cytochrome P450 metabolizing systems, and HO

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mRNA and enzyme activity are readily detectable in these cells [Mitani et al., 1990; Knowles et al., 1980]. Since heme is a natural endogenous regulator of cellular functions, we thought it important to determine the direct effect of heme (hemin) and other possible inducers on the expression of HO mRNA. Additionally, we evaluated the expression of HO mRNA and enzyme activity as a time-dependent phenomenon.

MATERIALS AND METHODS

Cell Cultures

Hep3B cells were obtained from American Type Culture Collection (Rockville, MD) and grown in 175 cm^2 Falcon tissue culture flasks (Becton-Dickinson, NJ) using Iscove's modified Dulbecco's medium (IMDM) (GIBCO, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 50 U penicillin/ml, 50 μg streptomycin/ml, and 2 mM glutamine. Cells were seeded into culture flasks at a concentration of 1×10^5 cells/ml and incubated at 37°C in a humidified 5% CO_2/95% air chamber. Old media was removed on day 3 and replaced with an equal volume of fresh IMDM. In some cases, the cultures were treated for various time periods with hemin (5–10 μM), CoPP (10 μM), zinc 2,4 bis glycol (10 μM), tin protoporphyrin (SnPP) (5–10 μM), dexamethasone (50 μg/ml), cycloheximide (1 μg/ml), actinomycin D (1 μg/ml), zinc protoporphyrin (ZnPP) (10 μM), poly I:C (50 μg/ml), and endotoxin (50 μg/ml).

Reagents

Guanidinium isothiocyanate and cesium chloride were purchased from Bethesda Research Laboratories (Gaithersburg, MD). SeaKem LE agarose was from FMC Corporation (Rockland, ME). Restriction endonucleases, Multiprime DNA labeling system, nitrocellulose, [α-^{32}P]dCTP, [α-^{32}P]UTP, and [α-^{35}S]dATP were from Amersham Corporation (Arlington Heights, IL). Co-protoporphyrin (CoPP) and all other protoporphyrins were from Porphyrin Products (Logan, UT). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO) or Fisher Scientific (Springfield, NJ).

$CoCl_2$ and hemin were prepared in buffer prior to the addition to cell cultures. CoPP was prepared under subdued light together with the appropriate amount of Tris base in 2 ml of 0.1 M NaOH with constant stirring. Thereafter, 1.2 ml of H_2O was added drop by drop, the pH adjusted to 7.8 with 0.1 N HCl, and the final volume brought up to 6 ml with H_2O. Hemin solution was prepared fresh each time in the same way as previously described [Abraham et al., 1982]. Briefly, 10 mg of hemin (Sigma) and 15 mg of Trizma base (Sigma) were dissolved in 2 ml of 0.1 N NaOH and the volume was brought to 10 ml by the addition of 7 ml of distilled water. The pH was adjusted to 7.8 by addition of 0.8–1.0 ml of 0.1 N HCl. The solution was kept in a dark bottle and diluted in PBS just before adding to culture.

cDNA Probe

The probe used was the 833-bp EcoRI/HindIII fragment prepared in pRHOI vector, a plasmid containing full-length cDNA for heme oxygenase [Shibahara et al., 1987]. The DNA fragment was obtained by electrophoresis in a low-gelling-temperature agarose gel and excision of the corresponding band as previously described [Abraham et al., 1991b]. The DNA was then labeled with [α-^{32}P] dCTP using the multiprime DNA labeling kit from Amersham to a specific activity of $1–2 \times 10^9$ cpm/μg. The probe was used at a concentration of 10^6 cpm/ml of the hybridization mixture [Lin et al., 1989].

In Situ Hybridization

The heme oxygenase cDNA was prepared as described above. A PBR322 cDNA probe (Boehringer-Mannheim) was used as a control. Probes were radiolabeled with [^{35}S]dATP using a random primer labeling protocol from Boehringer-Mannheim, to a specific activity of 1×10^5 cpm/ml. In situ hybridization was performed as described previously [Griffin et al., 1990]. Briefly, slides were rinsed in 2 × SSC, hybridized overnight at 37°C, soaked in increasingly stringent salt solutions, passed through an alcohol series, covered with Kodak NTB-2 liquid emulsion, and exposed for 7–10 days in the dark at 4°C. After development they were stained with freshly prepared Giemsa stain and examined under a light microscope. A cell was considered positive when silver grains over cells were 3× the background. Photomicrographs of cells are shown as figures.

Northern Blot Analysis of Total RNA

Total cellular RNA was extracted from a minimum of 5×10^7 cells/pellet by guanidinium isothiocyanate/$CsCl_2$ by the procedure of Chirg-

win et al. [1979]. Ten micrograms of Hep3B total RNA were denatured and size separated by electrophoresis at 100 V for 2.5 h in 1.5% (w/v) agarose/formaldehyde, and then blotted to nitrocellulose membranes (Amersham) for hybridization with a ^{32}P-labeled cDNA probe for human heme oxygenase [Shibahara et al., 1987]. Post hybridization washes were performed at 45°C in 1× SSC (150 mM NaCl/15 mM Na citrate) and 0.1% SDS. Binding of the heme oxygenase probe was detected by autoradiography.

Heme Oxygenase Assay

Microsomes were prepared as described elsewhere [Tenhunen et al., 1969]. The activity of microsomal heme oxygenase was determined using 0.5–1 mg protein in an incubation mixture described originally by Tenhunen et al. [1969]. Protein was determined by the method of Lowry et al. [1951] using bovine serum albumin as a standard.

RESULTS

Evaluation of Heme Oxygenase Expression by In Situ Hybridization and Total mRNA

Several agents were examined in Hep3B cultures for their ability to influence heme oxygenase mRNA. In situ hybridization and Northern blot analysis were utilized to assess the heme oxygenase mRNA accumulation in Hep3B cultures. Hemin was added to the Hep3B cells for various times from 5–20 min, and in situ hybridization was performed. As seen in Figure 1B, Hep3B cells cultured with hemin for as little as 5 min demonstrated a high number of heme oxygenase transcripts, as compared to untreated controls (Fig. 1A). After 20 min of exposure a significantly greater increase in heme oxygenase transcripts could be observed (Fig. 1C).

In the next set of experiments, we examined the effect of actinomycin D, an inhibitor of RNA synthesis, on the level of heme oxygenase in Hep3B exposed to hemin. Total RNA was extracted from these cultures and mRNA accumulation determined with ^{32}P-labeled heme oxygenase cDNA probe. RNA blot hybridization results are represented in Figure 2A. Heme oxygenase levels were greatly increased when hemin (lane 3) was added to cultures for 60 min prior to harvesting as compared to untreated cells (lane 1). The accumulation of heme oxygenase mRNA in Hep3B simultaneously exposed to hemin and actinomycin D was also examined. As seen in

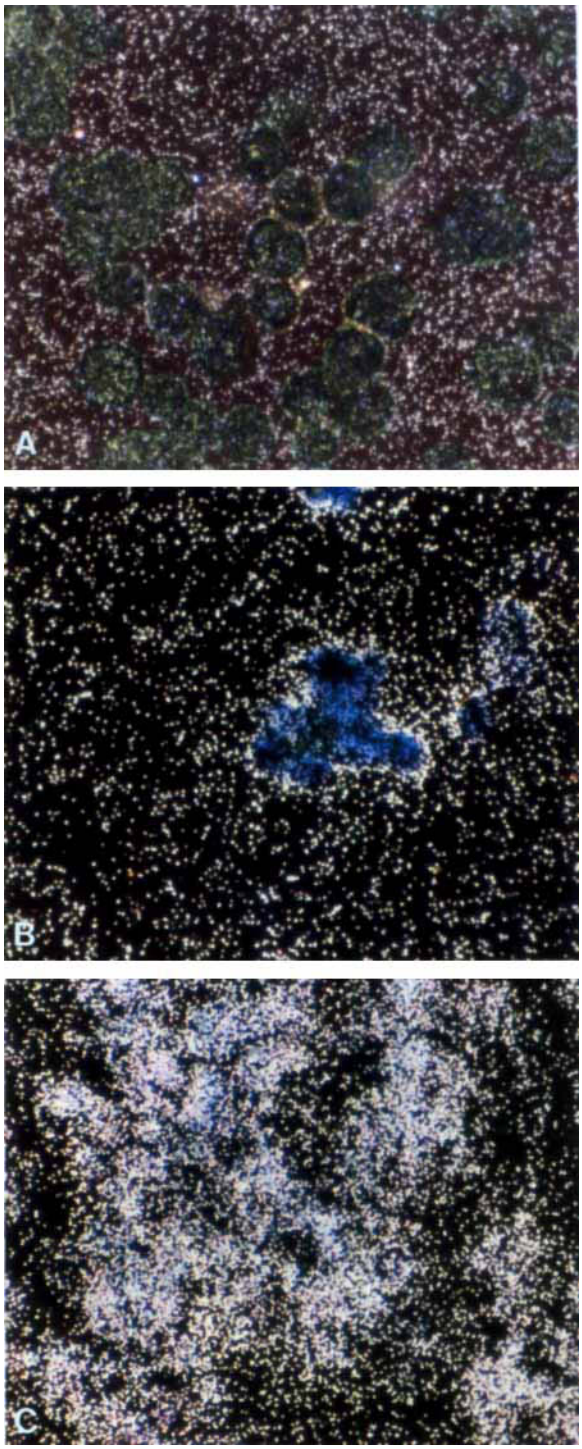

Fig. 1. Photomicrographs of Hep3B cells after in situ hybridization with heme oxygenase cDNA, using a dark field condenser at 500× magnification. **A:** Untreated Hep3B cells. **B:** Hep3B cells incubated with 10 μM hemin for 5 min. **C:** Hep3B cells incubated with 10 μM hemin for 20 min.

Figure 2A, the addition of actinomycin D (1 μg/ml) resulted in the suppression of heme oxygenase mRNA in Hep3B cultures incubated with hemin (lane 2). Quantitative evaluation of the intensity of the bands by scanning densitometry indicates that hemin causes accumulation of heme oxygenase mRNA by about 35-fold above the control. Hybridization of the filters with radiolabeled human γ-actin confirmed that similar amounts of total RNA were transferred to the filters in each lane of the paired samples (Fig. 2B).

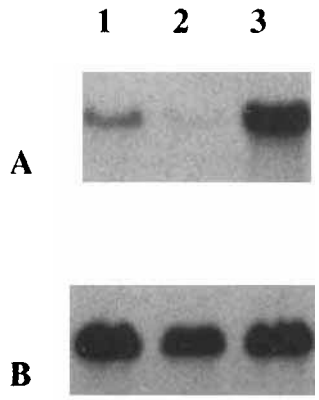

Fig. 2. Effect of hemin and actinomycin D on heme oxygenase mRNA expression in Hep3B 3-day cultures. Hep3B cells were cultured as indicated in the Methods section and treated for 1 h with the following: lane 1, untreated controls; lane 2, hemin (10 μM) + actinomycin D (1 μM/ml); lane 3, hemin (10 μM). RNA blot analysis was performed on total RNA of these cells. The heme oxygenase cDNA probe was nick-translated and hybridized to 10 μg of total cellular RNA prepared from Hep3B. RNA was prepared as described in Methods and Materials. RNA was hybridized with ^{32}P-labeled heme oxygenase cDNA as described in Methods. Identical blot was hybridized with ^{32} P-labeled human γ-actin cDNA (B). The blots were exposed for 16 h (**A**) and 2 h (**B**).

Effect of Porphyrins and Immune Modulators on Heme Oxygenase mRNA Expression

Several metalloporphyrins such as ZnPP, CoPP, and SnPP are well known to influence the activity of HO. We examined the effect of several metalloporphyrins and lipopolysaccharide (LPS), which is known to induce acute inflammatory reactions, on the level of heme oxygenase mRNA. Hep3B cells were exposed to the agents and the total RNA extracted after 1 h of exposure. The results are depicted in Figure 3 and show that most tested agents induced HO mRNA expression. Note that the HO mRNA bands for 10 μM ZnPP, CoPP, and SnPP exposed cells were much more intense than the control band (A). Also, the synthetic analogue, Zn 2,4 bis glycol deuteroprotoporphyrin (10 μM) and $CoCl_2$ (100 μM) were effective in inducing HO mRNA expression. Finally, the immune modulators LPS and PIC (50 μg) were both effective in inducing HO mRNA, with LPS being the most effective.

Effect of mRNA Inducing Agents on HO Activity

The direct effect of various inducing agents on HO activity was next examined. These results are represented in Table I. As expected, SnPP was a potent inhibitor of HO activity. This activity was suppressed by 95%, whereas LPS slightly enhanced HO activity even though both SnPP and LPS induced HO mRNA expression. In a similar manner, $CoCl_2$ caused an accumulation of heme oxygenase mRNA as well as enhancement of enzyme activity in the same as that for LPS. Activity values for $CoCl_2$ and LPS groups were 4.42 ± 0.40 and 4.76 ± 0.18 nM bilirubin/mg/h, respectively (Table I). Note on Table I that control HO activity was 2.06 ± 0.15 nM

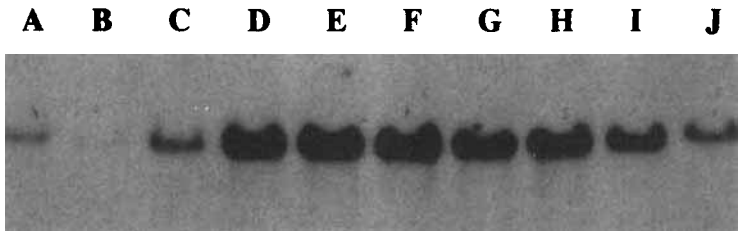

Fig. 3. Effect of porphyrins and immune modulators on heme oxygenase mRNA expression. Northern blot analysis of heme oxygenase mRNA. **Lane A:** Untreated control. **Lane B:** Dexamethasone. **Lanes C–E:** 10 μM ZnPP, CoPP, SnPP, respectively. **Lane F:** Zn 2,4 bis glycol (10 μM). **Lane G:** Hemin (10 μM). **Lane H:** Endotoxin (50 μg/ml). **Lane I:** Poly I:C (50 μg/ml). **Lane J:** $CoCl_2$ (100 μM). Exposure time for the autoradiogram was 24 h with an intensifying screen.

TABLE I. Effect of mRNA Inducing Agents on Heme Oxygenase Activity in Hep3B Cells*

Agent	Heme oxygenase activity nmole bilirubin/mg/h
Control	2.06 ± 0.15
$CoCl_2$ (200 μM)	4.42 ± 0.40
LPS (50 μg)	4.76 ± 0.18
SnPP (10 μM)	0.13 ± 0.02

*Agents were added to cultures containing 100 × 10^6 cells and incubated for 1½ h. Heme oxygenase activity was determined as described in Methods.

bilirubin/mg/h, whereas the level for SnPP exposed cells was only 0.13 ± 0.02 nM bilirubin/ng/h, or about 98% inhibition. Thus only SnPP inhibits HO activity, whereas the other agents tested resulted in enhancement of HO basal levels.

Time Course Induction of HO by Hemin

The next series of experiments was conducted in order to determine the effect of hemin on heme oxygenase mRNA as well as enzyme activity. Cells were incubated with 10 μM hemin for 0, 5, 10, 20, 40, and 60 min, after which the RNA was processed as described previously. Note in Figure 4 that elevated levels of HO mRNA are detected after only 5 min of hemin exposure (band A) as compared to control levels (band F). Furthermore, after rapid induction, elevated levels of HO mRNA are maintained throughout the following time periods, with maximal levels attained by about 40 min (band D).

In order to correlate the appearance of HO mRNA with the time necessary for detectable activity, time-dependent changes in HO enzyme activity were measured in response to hemin exposure (Fig. 5). As seen in Figure 5, the increase in activity of heme oxygenase was seen after about 40 min of hemin exposure, and a sustained increase in activity occurring after about 80 min of exposure. Maximal activity was then attained by 120–160 min.

We examined the differential effect of several unrelated structures on their ability to cause accumulation of heme oxygenase mRNA. The time course effect of hemin, LPS, and $CoCl_2$ for heme oxygenase mRNA induction was determined in order to detect the time required for induction by these agents. Total RNA was extracted from these cultures and mRNA accumulation determined using a ^{32}P-labeled heme oxygenase cDNA probe. RNA blot hybridization

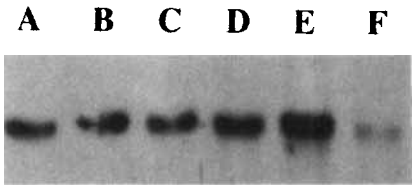

Fig. 4. Northern blot analysis of time-dependent effect of hemin on heme oxygenase mRNA induction. Hep3B cultures were exposed to hemin (10 μM) for 5–60 min. **Lane A:** 5 min. **Lane B:** 10 min. **Lane C:** 20 min. **Lane D:** 40 min. **Lane E:** 60 min. **Lane F:** Control, 0 min. Exposure time for the autoradiogram was 24 h with an intensifying screen.

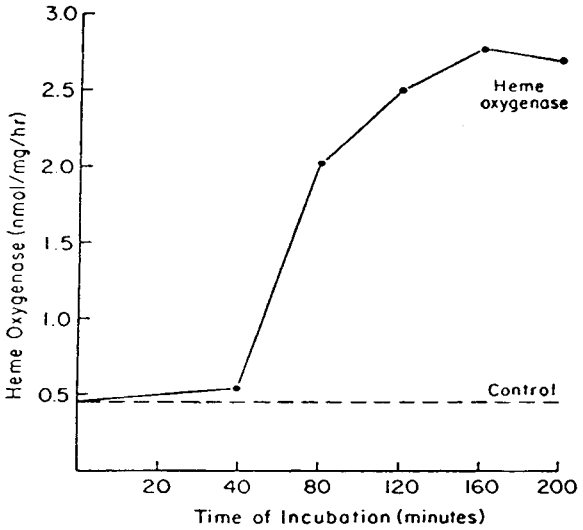

Fig. 5. Time-dependent changes in heme oxygenase activity in Hep3B cells in response to hemin exposure. Activity of heme oxygenase as a function of hemin exposure was examined. Enzyme activity is expressed as nmol of product per mg protein per h. Data are the results of two determinations.

was determined and autoradiographs scanned using a Beckman D7400. These results are represented on Figure 6 and show that hemin induces HO mRNA to near maximal levels within the first 10 min. LPS was less rapid in this induction as compared to hemin. Nevertheless, induction by LPS was quite effective within 20 min, whereas $CoCl_2$ remained near control levels at this time, but was followed by an increment in activity by 80 min. Thus, hemin promotes a very rapid induction in HO mRNA, and then returns to control levels by 24 h.

DISCUSSION

This study demonstrates that hemin is a rapid potent inducer of HO gene expression. Exposure of Hep3B cells to hemin for as little as 5 min resulted in the production of a significant number of HO transcripts as demonstrated by in situ

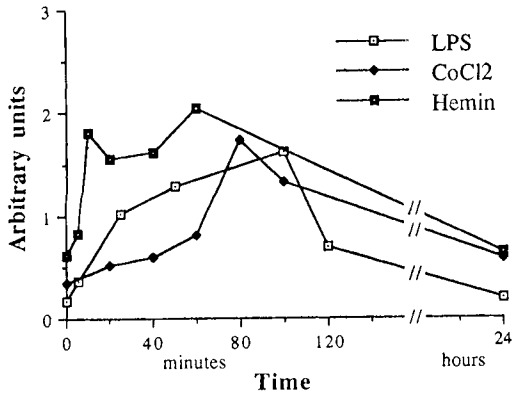

Fig. 6. Time course effect of hemin (10 μM) and lipopolysaccharide (LPS, 50 μg) and $CoCl_2$ (100 μM) on heme oxygenase mRNA expression in Hep3B cells. Results were obtained by densitometric scanning of Northern blot analysis.

hybridization. Furthermore, Northern analysis revealed enhanced expression of HO mRNA after only 5 min of hemin exposure. The induction of HO by hemin has been observed previously [Yoshida et al., 1988; Sassa et al., 1990]. However, the early time noted here needed for the induction of this process has not been reported until now. With the exception of LPS, such a rapid onset of HO gene expression was not as obvious with $CoCl_2$. Nevertheless, ZnPP, CoPP, SnPP, and $CoCl_2$ were all effective inducers of HO mRNA expression after 1 h of exposure, whereas LPS also induced enzyme activity and gave a more immediate response similar to hemin.

Direct measurements of microsomal HO activity revealed that after exposure of Hep3B cells to 10 μM hemin, HO activity could be detected after about 20 min. This period could represent the time needed for translational process of enzyme protein after the gene is expressed. Furthermore, SnPP was quite effective in inhibiting HO activity, even though HO mRNA was expressed. In contrast, LPS and $CoCl_2$ had no inhibitory effect on enzyme activity.

The rapid induction of HO mRNA by hemin and LPS suggests that these agents play a role in a natural immediate response mechanism that could benefit the cell during conditions of stress. The breakdown products (catabolism) of heme to porphyrin by-products (i.e. bilirubin) can act as powerful antioxidants against free radical-mediated injury [Stocker et al., 1987a,b]. In this respect, HO has been identified as a stress protein in a variety of tissues including brain and neoplastic cells [Ewing and Maines, 1991; Shibahara et al., 1987]. It should be recalled that LPS is a natural product of bacterial infection and is toxic to the host.

Previously, it was suggested that there are at least two mechanisms for the induction of heme oxygenase, namely, induction by a heat shock transcription factor and by a putative heme responsive transcription factor [Abraham et al., 1991b]. In this respect, $CoCl_2$ induces HO through a HO transcriptional factor (HOTF) on the promotor region, while heme involves other motifs. HOTF binds to the promotor region (−50/−35) of rat HO and stimulates in vitro transcription of the HO gene [Abraham et al., 1991b].

The different pattern of induction of HO mRNA by LPS as contrasted with $CoCl_2$ suggests that LPS may act through a different transcriptional factor than those involved with $CoCl_2$, but it may be similar to heme. It also remains possible that an agent such as LPS may induce membrane lipid peroxidation and free radical formation [Kikkawa et al., 1984] which in turn causes the release of heme and subsequent stimulation of HO. Thus, the induction of HO may serve as an important acute phase protein under conditions of stress.

ACKNOWLEDGMENTS

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