Heme Oxygenase Is a Positive Acute-Phase Reactant in Human Hep3B Hepatoma Cells

By Kinuko Mitani, Hiroyoshi Fujita, Attallah Kappas, and Shigeru Sassa

The effects of human interleukin-6 (hIL-6), the major acute-phase inducer, on the level of the transcript of microsomal heme oxygenase (HO) were examined in a human hepatoma cell line, Hep3B. Messenger RNAs (mRNAs) encoding HO and haptoglobin (Hpt) increased after hIL-6 treatment in a time- and dose-dependent manner. hIL-6 had no effect on the induction of heat-shock protein 70 (hsp70) mRNA, suggesting that the induction of HO by hIL-6 is regulated by a different mechanism from that which mediates the heat-shock induction of this enzyme. The hIL-6–mediated induction of HO mRNA was completely abrogated by simultaneous treatment of cells with actinomycin D, but not with cycloheximide, suggesting that the induction occurs at the level of transcription. A nuclear factor was shown both in untreated, and in the hIL-6–treated Hep3B cells that binds specifically to the IL-6–responsive element (IL6-RE) of the human HO gene. These findings suggest that HO is a positive acute-phase reactant in this human liver-derived cell line, and that the nuclear factor specific to the IL6-RE may be involved in the activation of the HO gene after hIL-6 treatment.

MICROSOMAL heme oxygenase (EC 1.14.99.3) (HO) is the rate-limiting enzyme in the oxidative catabolism of heme that yields biliverdin IXα.[1] The activity and the transcript of HO can be increased in many cell types by treatment with hemin, the substrate for the enzyme, as well as with various other nonheme substances.[2] Recently, HO has been reported to be a major 32-Kd stress protein as well as a heat-shock protein (hsp) in various cell types.[3-6] It is known that endotoxin, a bacterial lipopolysaccharide, increases HO activity in the rat liver.[7] Endotoxin is also involved in the induction of an acute-phase reaction that occurs in response to inflammation and is characterized by increased hepatic synthesis and secretion of a variety of secretory proteins.[8] Thus, it appears that HO gene activation may also occur during an acute-phase reaction in the human liver. To study this question, we used a human hepatoma cell line, Hep3B, which is known to express a well differentiated hepatocyte phenotype,[9] and recombinant human interleukin-6 (hIL-6), which is the major monokine involved in the induction of an acute-phase reaction.[10] Our results indicate that HO messenger RNA (mRNA) is induced at the level of transcription in association with the induction of an acute-phase reaction.

MATERIALS AND METHODS

Cell culture and treatment with hIL-6. Hep3B cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultivated as described previously.[3] To treat cells with hIL-6 for various time periods, cells were incubated with the growth medium containing hIL-6, which was replenished after 2 days of incubation. To treat cells with various concentrations of hIL-6, or with actinomycin D or cycloheximide, cells were incubated for 6.5 hours with the chemicals.

Northern blot analysis. Total RNA was isolated from 1×10^7 cells and Northern blot analysis was performed as described previously.[3] Data were expressed as the ratio of the level of mRNA in the treated cells to that in the untreated control.

cDNA probes. Probes used were human HO (pHHO1), human albumin cDNA, human haptoglobin (Hpt) cDNA, and human hsp70 (pH2.3).[3,6] Each cDNA was inserted into p-GEM4z vector (Promega Biotech, Madison, WI) for the transcription of an RNA probe, as described previously.[3]

Gel retardation assay. Gel mobility-shift DNA-binding assays were performed as described previously.[11]

RESULTS

Time-dependent accumulation of HO and Hpt mRNAs after hIL-6 treatment. After treatment of cells with hIL-6 (100 U/mL), the levels of HO mRNA increased up to approximately twofold greater than the control level within 6 hours of treatment (Fig 1A). A maximal level of accumulation of HO mRNA (fivefold of the untreated control) was observed after treatment for 48 hours, or thereafter. hIL-6 treatment also resulted in a significant induction of Hpt mRNA, starting 2 hours after treatment (approximately ninefold over the control), and a maximal level (approximately 25-fold over the control level) was observed at 48 hours (Fig 1B).

Dose-dependent changes in the levels of mRNAs for HO, hsp70, Hpt, and albumin after hIL-6 treatment. Figure 2 shows the effects of hIL-6 treatment on the levels of mRNAs encoding HO, hsp70, Hpt, and albumin in Hep3B cells. When Hep3B cells were incubated for 6.5 hours with various concentrations of hIL-6, HO mRNA increased in a dose-dependent manner, starting from 10 U/mL up to 50 U/mL. The maximal level of the mRNA at 50 U/mL hIL-6 was 2.1-fold greater than the untreated control, while higher concentrations had a lesser effect. In contrast, the levels of hsp70 mRNA were not influenced at all concentrations of hIL-6 (Fig 2A). hIL-6 treatment caused a marked increase in the level of Hpt mRNA (~44-fold increase over the control), while it decreased that of albumin mRNA

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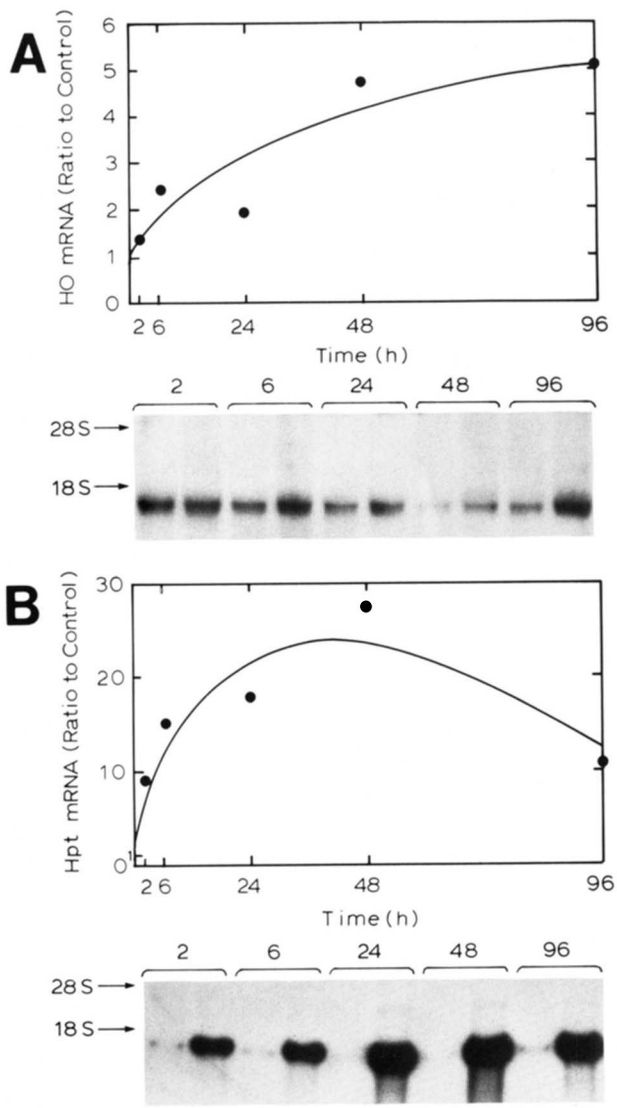

Fig 1. Time courses of HO mRNA (A) and Hpt mRNA (B) accumulation in Hep3B cells after hIL-6 treatment. Untreated (left) and the hIL-6–treated (right) RNA samples are shown in each pair.

(70% less than the control), even at the lowest concentration examined, ie, 10 U/mL (Fig 2B).

Blockade of the hIL-6–mediated accumulation of HO mRNA by actinomycin D, but not by cycloheximide. When actinomycin D, a specific inhibitor of DNA-directed RNA synthesis, was added to Hep3B cells at the beginning of hIL-6 treatment, the hIL-6–mediated accumulation of HO mRNA was essentially abolished (Fig 3), while treatment with cycloheximide, an inhibitor of protein synthesis, had no effect. These findings suggest that the accumulation of HO mRNA is dependent on de novo RNA synthesis.

A nuclear factor that binds specifically to the IL-6–responsive element (IL6-RE) of the human HO gene in Hep3B cells. Gel retardation assays using extracts from untreated or the hIL-6–treated Hep3B cells showed the formation of an IL6-RE–specific DNA-protein complex with oligonucleotides A and B (Fig 4A) that contained the IL6-RE of the human HO promoter (Fig 4B). Among several bands observed, a band, termed 'IL6', was specific to the oligonucleotide B that contained only the IL6-RE, but not the heat-shock element (HSE), while other complexes were commonly observed by the two oligonucleotides. It should be noted that the band was expressed constitutively both in untreated and the hIL-6–treated cells. Inhibition of the DNA-protein complex formation with the radiolabeled oligonucleotide B was also examined by adding unlabeled oligonucleotide A, B, D, or DNA fragment C to the reaction mixture. Oligonucleotide B (Fig 4B) and fragment C (Fig 4C), containing the IL6-RE in a reverse orientation, significantly blocked the complex formation with the radiolabeled oligonucleotide B. Oligonucleotide A that contained both IL6-RE and HSE competed with the complex formation, but with a weaker effect than oligonucleotide B or fragment C (Fig 4C). Oligonucleotide D, lacking the IL6-RE, had little effect on the specific complex formation (Fig 4C). These findings suggest that there is a nuclear factor(s) specific to the IL6-RE sequence in the human HO gene in Hep3B cells.

DISCUSSION

The results of the present study provide evidence for the induction of HO mRNA in Hep3B cells after treatment with hIL-6. The kinetics of accumulation of mRNAs encoding HO and Hpt were similar, although the magnitude of accumulation was much greater in the case of Hpt mRNA (Figs 1B and 2B). The induction was maximum at a hIL-6 concentration of 50 U/mL when cells were incubated for 6.5 hours (Fig 2A). Because there was a decrease in albumin mRNA, a negative acute-phase reactant[10] (Fig 2B), while there was an increase in Hpt mRNA, a positive acute-phase reactant[10] in the hIL-6–treated cells (Figs 1B and 2B), it is clear that an acute-phase reaction was induced in these cells. Thus, the induction of HO mRNA can be considered as an event associated with the acute-phase reaction. The hIL-6–mediated induction of HO was essentially abolished by simultaneous treatment of cells with actinomycin D (Fig 3), suggesting that the induction occurs at the level of transcription. The induction of HO by hemin,[12] heat-shock,[3,13,14] heavy metals,[12,14-16] and cobalt protoporphyrin[17] has also been shown to be transcriptional.

Both HO[18] and hsp70[19] genes contain a common HSE in the 5′-flanking region (Fig 4A), and both mRNAs are inducible with heat shock in Hep3B cells.[3,6] Our findings show that hIL-6 is an inducer of HO mRNA, but not of hsp70 mRNA (Fig 2A). This finding suggests that the induction of HO mRNA by hIL-6 is not mediated by HSE. The promoter region of the human HO gene contains two IL6-RE sequences, ie, TTCTGGGAC, nucleotide residues −373 to −365, and AAGGGTCAT, nucleotide residues −160 to −152 (the latter is in a reverse orientation),[20] which share significant homology with the consensus sequence ([T/A]T[C/G]TGGGA[A/T]) found in the potentially regulatory region of various acute-phase protein genes,[21,22] while a similar sequence has not been reported in the

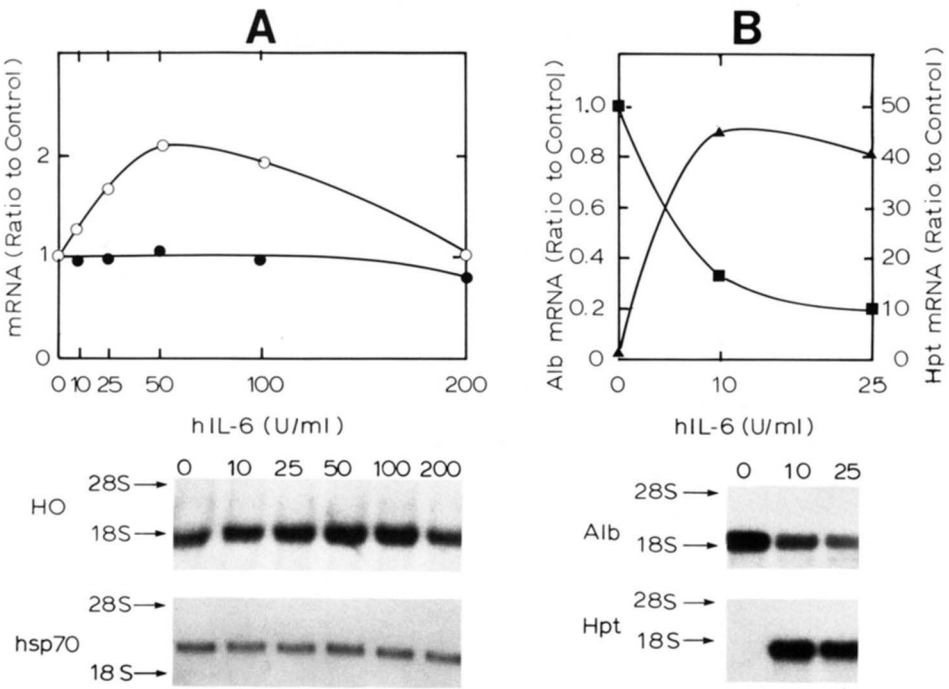

Fig 2. Effects of various concentrations of hIL-6 treatment on mRNAs encoding HO, hsp70, Hpt, and albumin in Hep3B cells. Cells were incubated with hIL-6 for 6.5 hours. (A) (○) HO; (●) hsp70. (B) (▲) Hpt; (■) albumin.

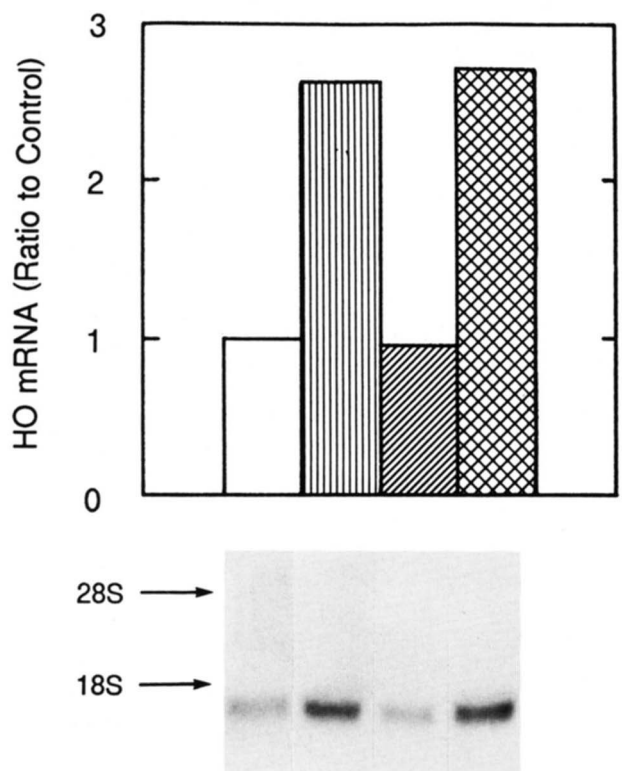

Fig 3. The effect of actinomycin D and cycloheximide on the hIL-6–mediated increase in HO mRNA in Hep3B cells. Cells were incubated for 6.5 hours. (□) No treatment; (▨) hIL-6 100 U/mL; (▨) hIL-6 100 U/mL + actinomycin D (0.25 μg/mL); (▩) hIL-6 100 U/mL + cycloheximide (1 μg/mL).

5′-flanking region of the human hsp70 gene.[19] This sequence has a crucial role in the induction of rat α_2-macroglobulin gene in an acute-phase reaction.[22] Our findings also show the formation of a nuclear factor-DNA complex specific to the IL6-RE in the human HO promoter in Hep3B cells. It should be noted that the nuclear factor specific to the IL6-RE of the HO gene is constitutively expressed also in untreated Hep3B cells. This situation is similar to the constitutive expression of another transcriptional factor, the nuclear factor for IL-6 expression (NF-IL6), in Hep3B cells, which binds to an IL-1–responsive element in the IL-6 gene.[23] Thus, it is tempting to speculate that hIL-6 may activate the human HO gene via the IL6-RE, in a manner similar to that which involves NF-IL6.

Our findings suggest that the increase of HO mRNA concentration may serve an indicator of an acute-phase reaction. Although acute-phase reactants are generally believed to be involved in the host-defence response,[10,24] their exact role is not well understood. HO is unique in that its function as an enzyme to catalyze the oxidative breakdown of heme is well defined. Free heme is a potent pro-oxidant, while bile pigments, the products of the HO reaction, are physiologically important anti-oxidants.[25] As such, induction of HO can provide protection against the potentially toxic effects of the heme-mediated oxidative stress.[25] In this respect, it is of interest to note that there is, as yet, no known genetic deficiency of HO described, while genetic defects of each enzyme in the heme biosynthetic pathway have been recognized. It is possible, therefore, that HO may be important in fundamental cellular processes,[17,25,26] such as the acute-phase reaction, and that a

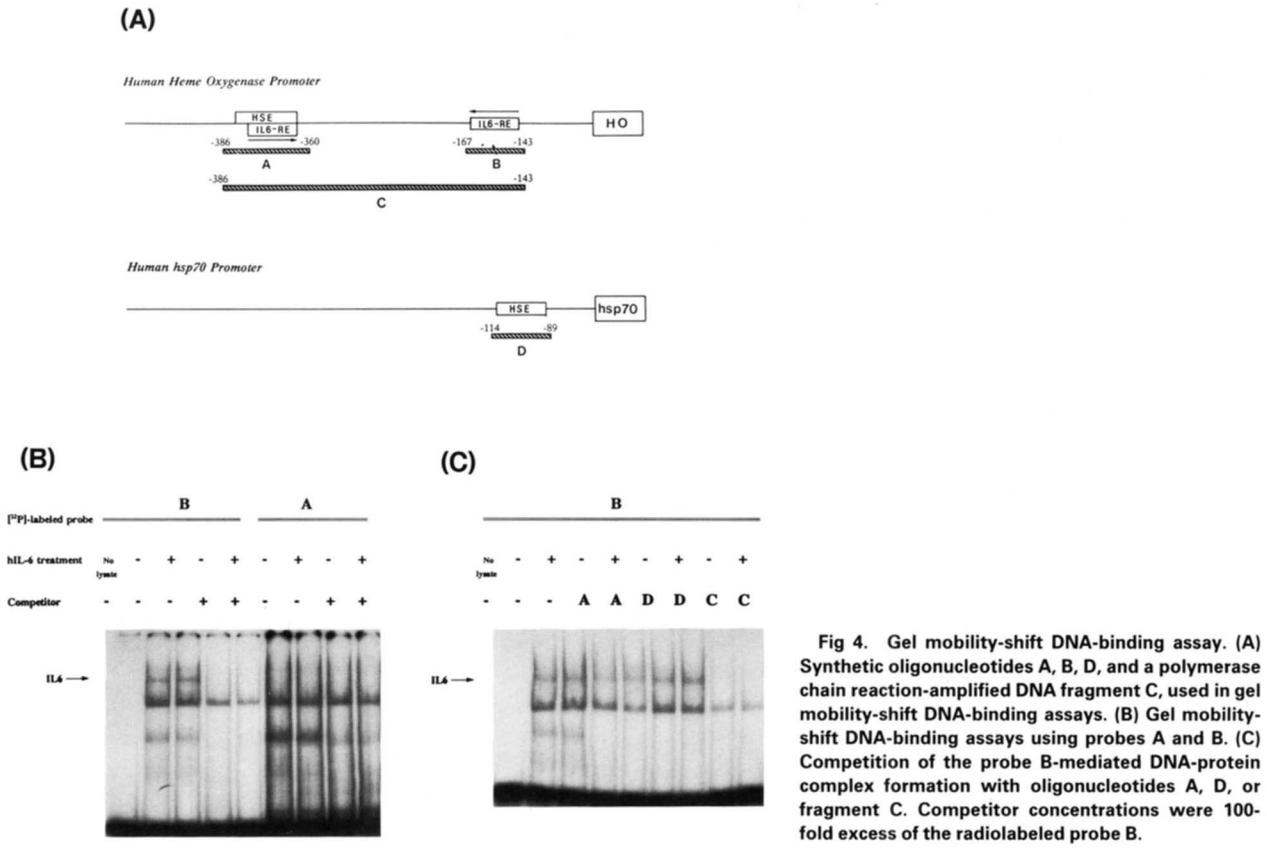

Fig 4. Gel mobility-shift DNA-binding assay. (A) Synthetic oligonucleotides A, B, D, and a polymerase chain reaction-amplified DNA fragment C, used in gel mobility-shift DNA-binding assays. (B) Gel mobility-shift DNA-binding assays using probes A and B. (C) Competition of the probe B-mediated DNA-protein complex formation with oligonucleotides A, D, or fragment C. Competitor concentrations were 100-fold excess of the radiolabeled probe B.

genetic deficiency of this enzyme may be deleterious to the host.

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