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SUPPRESSION OF CYTOCHROME P450IA1 BY INTERLEUKIN-6 IN HUMAN HepG2 HEPATOMA CELLS

YOSHIAKI FUKUDA and SHIGERU SASSA*
The Rockefeller University, New York, NY 10021, U.S.A.

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Abstract—The effects of interleukin-6 (IL-6), the major inducer of the acute-phase reaction, on the expression of cytochrome P450IA1 (CYPIA1) were examined using human HepG2 hepatoma cells. Treatment of cells with IL-6 decreased the level of 3-methylcholanthrene-induced CYPIA1 protein and its mRNA. Nuclear runoff analysis revealed that the effect of IL-6 was largely transcriptional. IL-6 treatment of HepG2 cells increased mRNA for microsomal heme oxygenase, the rate-limiting enzyme in heme catabolism, suggesting that the suppressive effect of IL-6 on CYPIA1 mRNA may be due to a loss of heme. Consistent with this hypothesis, simultaneous treatment of cells with Sn-mesoporphyrin, an inhibitor of heme oxygenase, prevented the IL-6-mediated suppression of CYPIA1. These findings suggest that the suppression of P450IA1 mRNA by IL-6 appears to occur, at least in part, from the decline in free heme content as a result of the induction of heme oxygenase. Our results raise the possibility that other physiological as well as environmental stimuli which affect cellular heme concentrations may also modulate the expression of P450s.

Key words: acute-phase reaction; cytochrome P450; heme oxygenase; interleukin-6; HepG2 cells; tin-mesoporphyrin

Cytochrome P450s constitute a superfamily of hemeproteins that are highly expressed in the liver of animals and humans and play an essential role in the metabolism of endogenous compounds such as steroid hormones, biliary salts, and fatty acids, and in the detoxification of xenobiotic molecules including drugs, environmental pollutants, and carcinogens [1–3]. Cytochrome P450s are also subject to induction or suppression by exposure of the host to various chemicals [3]. The extent of such responses may likely be determined by the availability of heme, since as much as 65% of heme produced in the liver is utilized for the formation of cytochrome P450s [4].

HepG2 human hepatoma cells are known to retain and express many properties that are characteristic of normal hepatic parenchymal cells [5, 6]. Data from our laboratory [7, 8] and from others [9] demonstrated that these cells maintain active heme biosynthesis, contain measurable levels of cytochrome P450s, and show induction responses of cytochrome P450s. These findings suggest that HepG2 cells may offer a useful model system for the study of experimental manipulation of human cytochrome P450s.

The acute-phase reaction is characterized by profound changes in hepatic synthesis and circulating concentrations of plasma protein which occur in response to infections and tissue injuries [10]. The proteins induced by an acute-phase reaction are thought to play an important role in host defense by acting as opsonins, protease inhibitors, or carriers of various hormones [11]; in addition, they may also influence other biological processes such as cytochrome P450s and their associated hydroxylase activities. It is known that induction of the acute-phase reaction by treatment of animals with endotoxin results in a decreased level of hepatic cytochrome P450s [12, 13]. Although it is conceivable that a similar change may also occur in humans during infection, little is known about changes in cytochrome P450s during the human acute-phase reaction.

In this study, the effect of IL-6,† the major acute-phase inducer [14], on the expression of cytochrome P450IA1 (CYPIA1) and its mRNA was examined in HepG2 cells. Our results indicate that IL-6 significantly suppresses the CYPIA1 protein and its mRNA. We also demonstrate that the suppressive effect of IL-6 on CYPIA1 is probably mediated by the induction of microsomal heme oxygenase, the rate-limiting enzyme in heme catabolism.

MATERIALS AND METHODS

Cell cultures and treatment with chemicals. HepG2 cells were a gift from Dr. Barbara B. Knowles, Wistar Institute, Philadelphia, PA. Cells were cultivated as described previously [15]. 3MC (Sigma Chemical Co., St. Louis, MO) was dissolved in dimethyl sulfoxide at a concentration of 6 mM, and added to culture to yield a final concentration of 6 μM. IL-6 was a gift from either Genetics Institute, Boston, MA, or the Kirin Brewery Co., Ltd., Maebashi, Japan.

Probes. cDNA for human CYPIA1 (phP1-450-3′)

* Corresponding author: Shigeru Sassa, M.D., Ph.D., The Rockefeller University, New York, NY 10021. Tel. (212) 327-8497; FAX (212) 327-8872.

† Abbreviations: ALA, δ-aminolevulinate; CYP, cytochrome P450; IFN, interferon; IL-6, interleukin-6; 3MC, 3-methylcholanthrene; and SnMP, Sn-mesoporphyrin.

[16] was obtained from the American Type Culture Collection (Rockville, MD). A 1046 bp fragment (1521 to 2567) of CYPIA1 cDNA was inserted into pGEM3Z vector (Promega Corp., Madison, WI). A radioactive RNA probe was synthesized according to the method of Melton *et al.* [17] for northern blot analysis. Human β-actin cDNA was purchased from CLONTECH Laboratories, Inc. (Palo Alto, CA), and labeled by nick translation.

Northern blot analysis. Total RNA was isolated according to the method of Cathala *et al.* [18]. RNA (15 μg) was applied to a 1.2% agarose/formaldehyde gel, electrophoresed, and transferred to a sheet of Zeta-probe filter (Bio-Rad, Richmond, CA). The filters were hybridized with an RNA probe for CYPIA1 at 50° for 24 hr in a solution containing 50% formamide, 1.5× SSPE (270 mM NaCl, 15 mM Na_2HPO_4, 1.5 mM EDTA), 1% SDS, 0.5% BLOTTO, 0.2 mg/mL yeast transfer RNA, 0.5 mg/mL sonicated salmon sperm DNA. After hybridization, filters were treated with 1 μg/mL RNase A for 10 min at room temperature, and were washed in 0.1× SSC (SSC = 0.15 M sodium chloride + 0.015 M sodium citrate) for 30 min at 65°. The level of β-actin mRNA was also examined as a control. mRNA concentrations were determined by an LKB Ultroscan XL densitometer (LKB Pharmacia, Uppsala, Sweden). There was a linear relationship between the amount of mRNA and the absorbance at 633 nm. Experiments were repeated at least twice using separate preparations of RNA, and similar results were observed in each experiment. Since there were no major significant changes in β-actin mRNA levels, results for P450IA1 and β-actin were

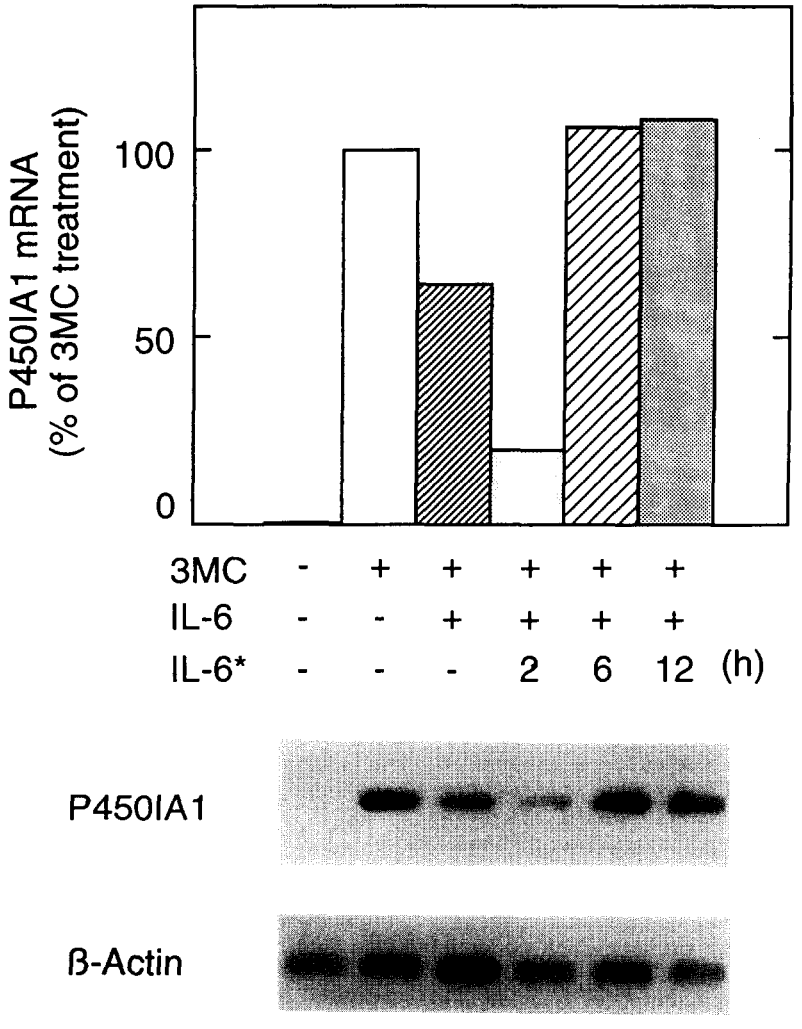

Fig. 1. Effect of IL-6 on the expression of CYPIA1 mRNA induced by 3MC in HepG2 cells. Cells were incubated in the presence or absence of 3MC (6 μM) and IL-6 (100 U/mL) for 48 hr. In addition, groups marked as "IL-6*" were preincubated with IL-6 (100 U/mL) for 2, 6, or 12 hr. Cells were harvested 48 hr after the addition of 3MC. Total RNA was examined by northern blot analysis using the probe for CYPIA1, as described in Materials and Methods. The level of β-actin mRNA was also examined as a control. Data are expressed as percent of the value of the 3MC-treated culture.

expressed for each mRNA, without normalization based on β-actin mRNA content.

Western blot analysis. Microsomes were isolated from 1×10^8 cells as described previously [7]. Microsomal proteins were electrophoretically separated using a 10% polyacrylamide gel including SDS. Immunochemical detection of CYPIA1 was performed using a rabbit polyclonal antibody against rat liver microsomal CYPIA1 (OXYgene, Dallas, TX), a peroxidase-coupled goat anti-rabbit IgG as the second antibody, and an enhanced chemiluminescence assay system (Amersham Corp., U.K.) as described previously [19].

Nuclear runoff transcription analysis. Nuclei from 5×10^7 cells were lysed in a solution of 10 mM Tris–HCl (pH 7.5), 10 mM NaCl, 2 mM $MgCl_2$, and 0.1% NP-40, and resuspended in a nuclear freezing buffer [40% glycerol, 50 mM Tris–HCl (pH 8.3), 5 mM $MgCl_2$, and 0.1 mM EDTA], and stored at −80°. The nuclear runoff reaction was performed according to the method described by Linial *et al.* [20]. In brief, a nuclear lysate equivalent to 2×10^7 nuclei

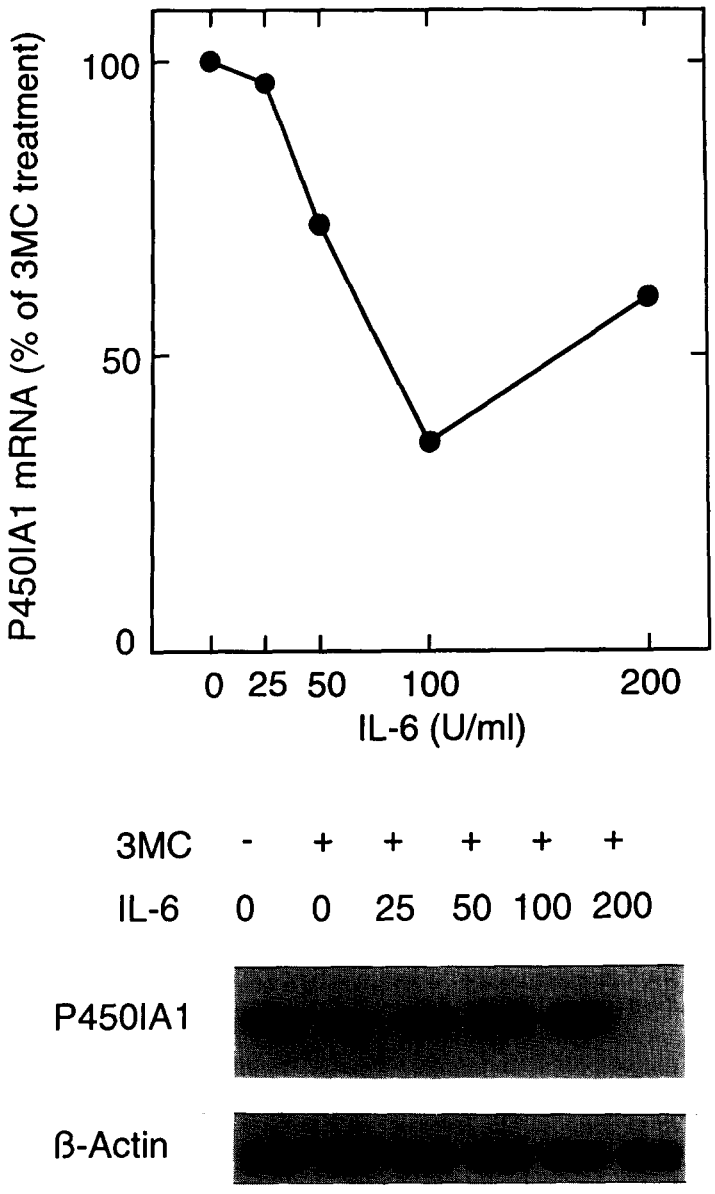

Fig. 2. Concentration–response effects of IL-6 on the expression of CYPIA1 mRNA induced by 3MC. HepG2 cells were incubated in the presence or absence of 3MC (6 μM), with various concentrations of IL-6 (100 U/mL). IL-6 was added 2 hr prior to the addition of 3MC. Cells were harvested 48 hr after the addition of 3MC, and total RNA was examined by northern blot analysis using the probe for CYPIA1, as described in Materials and Methods. The level of β-actin mRNA was also examined as a control. Data are expressed as percent of the value of the 3MC-treated culture.

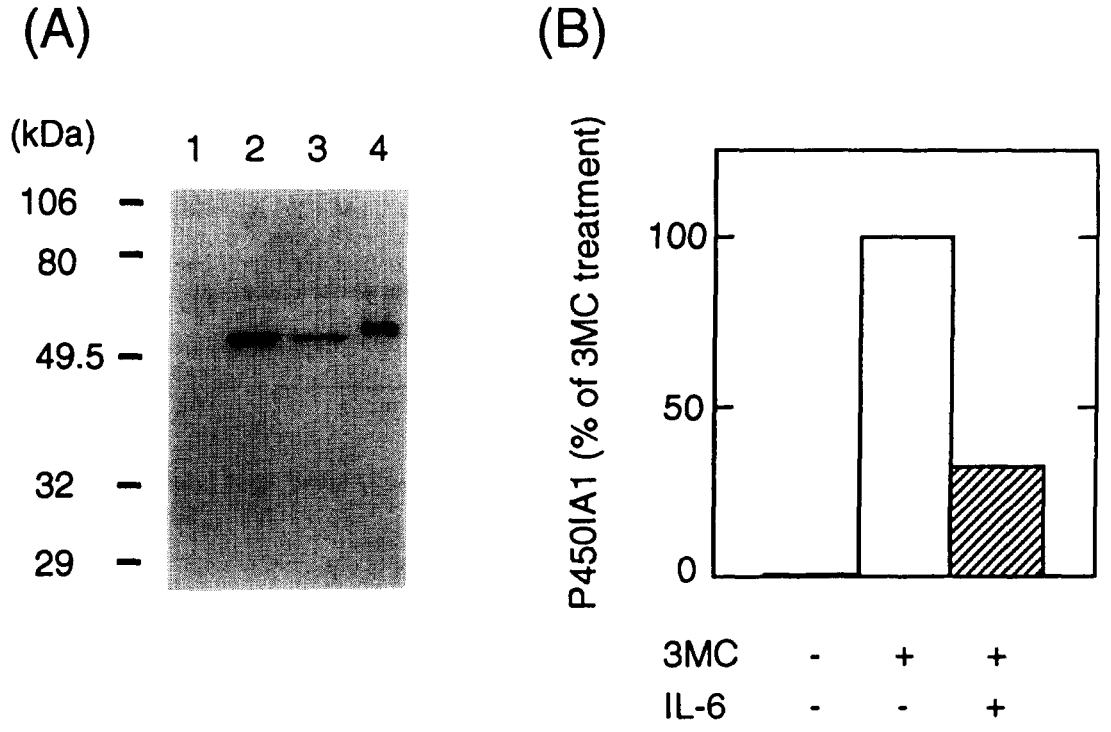

Fig. 3. Effect of IL-6 on the expression of the CYPIA1 protein induced by 3MC. HepG2 cells were incubated in the presence or absence of 3MC (6 μM), with or without IL-6 (100 U/mL). IL-6 was added 2 hr prior to the addition of 3MC. Cells were harvested 48 hr after the addition of 3MC, and microsomal proteins were analyzed by western blot analysis, as described in Materials and Methods. (A) Lane 1, untreated; Lane 2, treated with 3MC; Lane 3, treated with 3MC and IL-6; Lane 4, purified rat microsomal CYPIA1. Molecular masses are indicated on the left. (B) Densitometric quantitation of the human CYPIA1 protein. Data are expressed as percent of the value of the 3MC-treated culture.

was incubated in a final volume of 400 μL reaction buffer, containing 5 mM Tris–HCl (pH 8.0), 2.5 mM $MgCl_2$, 150 mM KCl, 0.25 mM ATP, GTP, and CTP, and 7.4 mBq of [α-^{32}P]UTP (New England Nuclear, Boston, MA) for 30 min at 30°. The ^{32}P-labeled RNA was purified through a G-50 spin column, and the labeled RNA (5×10^6 cpm/mL) was hybridized to nitrocellulose filters containing 5 μg of linearized plasmid DNAs. After hybridization, filters were washed for 1 hr in 2× SSC at 65°. The filters were then incubated at 37° with RNase A (10 μg/mL) for 30 min and subsequently washed in 2 × SSC for 1 hr. RNA concentrations were determined from autoradiograms by densitometry.

RESULTS

Effect of IL-6 on CYPIA1 mRNA. The level of mRNA encoding CYPIA1 in human HepG2 hepatoma cells was examined following treatment with 3MC, in combination with IL-6. CYPIA1 mRNA was undetectable in untreated HepG2 cells, whereas it was increased markedly by treatment with 3MC (Fig. 1). The combined addition of 3MC and IL-6 substantially decreased the 3MC-induced CYPIA1 mRNA level (49.8 ± 19.6% of the 3MC control, mean ± SEM, N = 4). When added 2 hr prior to 3MC, IL-6 exerted even a greater suppressive effect on CYPIA1 mRNA (29.0 ± 8.2% of the 3MC control, N = 2), while no significant suppression was observed when IL-6 was added 6 hr, or 12 hr prior to 3MC.

The effect of various concentrations of IL-6 on CYPIA1 mRNA is shown in Fig. 2. IL-6 suppressed the level of CYPIA1 mRNA in a concentration-dependent manner, with a maximum suppression occurring at 100 U/mL.

Effect of IL-6 on the CYPIA1 protein. Western blot analysis was performed to examine the effect of IL-6 on the expression of the CYP1A1 protein. As shown in Fig. 3, there was no immunochemically detectable human CYPIA1 in untreated HepG2 cells. In contrast, 3MC treatment induced a protein corresponding to the M_r of the human CYPIA1 (58 kDa [21]). The human protein migrated slightly faster than the purified rat CYPIA1 (Fig. 3A, lanes 2–4), consistent with it being 12 amino acids smaller [21, 22]. Treatment of cells with IL-6 significantly decreased the CYPIA1 protein (34% of the 3MC control).

Effect of IL-6 on the rate of transcription of the CYPIA1 gene. To examine the effect of IL-6 on the transcription of the CYPIA1 gene in HepG2 cells, *in vitro* nuclear runoff transcription assays were

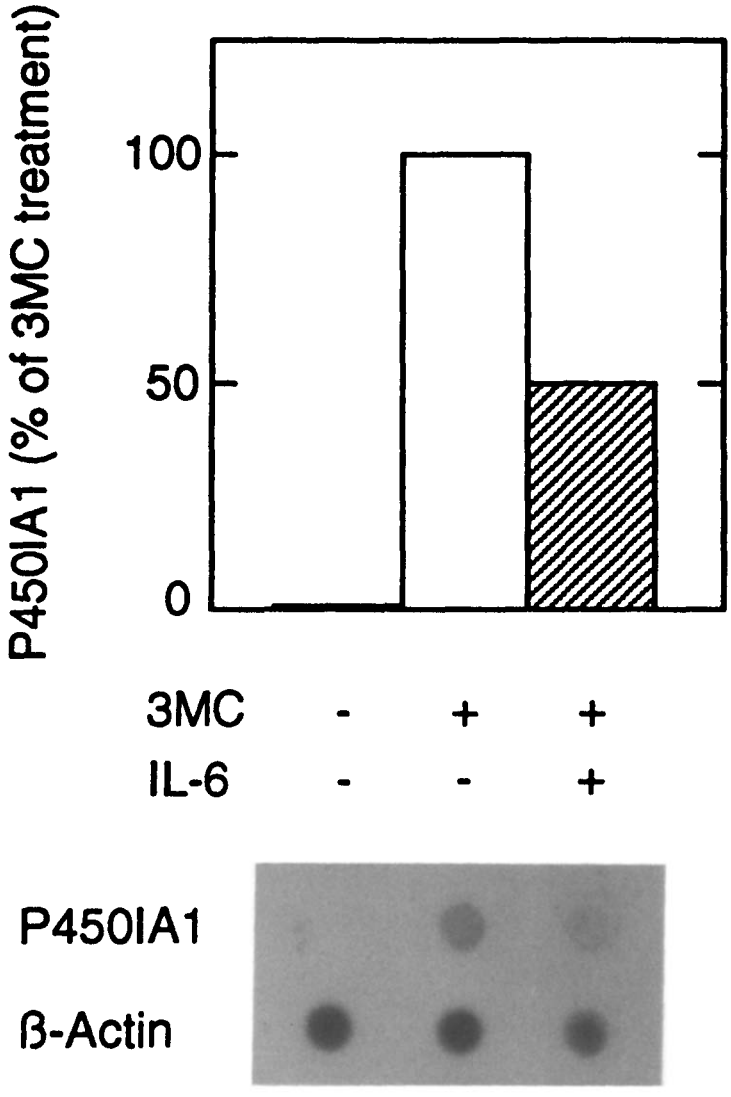

Fig. 4. Effect of IL-6 on the transcriptional rate of the CYPIA1 gene. HepG2 cells were incubated in the presence or absence of 3MC (6 μM), with various concentrations of IL-6 (100 U/mL). IL-6 was added 2 hr prior to the addition of 3MC. Cells were harvested 12 hr after the addition of 3MC, and a runoff transcription assay was performed, as described in Materials and Methods. Dot blots were quantitated by densitometry, and data are expressed as percent of the value of the 3MC-treated culture.

performed. Treatment of HepG2 cells with 3MC resulted in a substantial increase in CYPIA1 transcription (Fig. 4). IL-6 treatment markedly suppressed the 3MC-induced CYPIA1 transcription (Fig. 4). These findings were reproduced in two separate experiments (48.1% in Expt 1, and 54.8% in Expt 2) and suggest that the increase of CYPIA1 mRNA by 3MC treatment and the suppression of CYPIA1 mRNA by IL-6 (Figs. 1 and 2) largely occur at the transcriptional level.

Effect of IL-6 on heme oxygenase mRNA. Changes in the level of mRNA encoding heme oxygenase were also examined in HepG2 cells following treatment of cells with IL-6. Heme oxygenase mRNA was detectable in untreated HepG2 cells, and its level increased rapidly after IL-6 treatment (1.4-fold at 30 min), and continued to increase for at least 12 hr (Fig. 5); these findings are consistent with our earlier data [23]. The induction of heme oxygenase activity by various treatments in a number of systems has been shown to be transcriptional [24]. Our own findings also indicate that IL-6 increases heme oxygenase by a transcriptional mechanism [23].

Effect of SnMP on CYPIA1 mRNA. To investigate whether the induction of heme oxygenase is involved in the down-regulation of CYPIA1, cells were incubated with SnMP, a specific inhibitor of heme oxygenase activity [25]. The addition of IL-6 suppressed the 3MC-induced CYPIA1 mRNA (37.1% of the 3MC control), while the addition

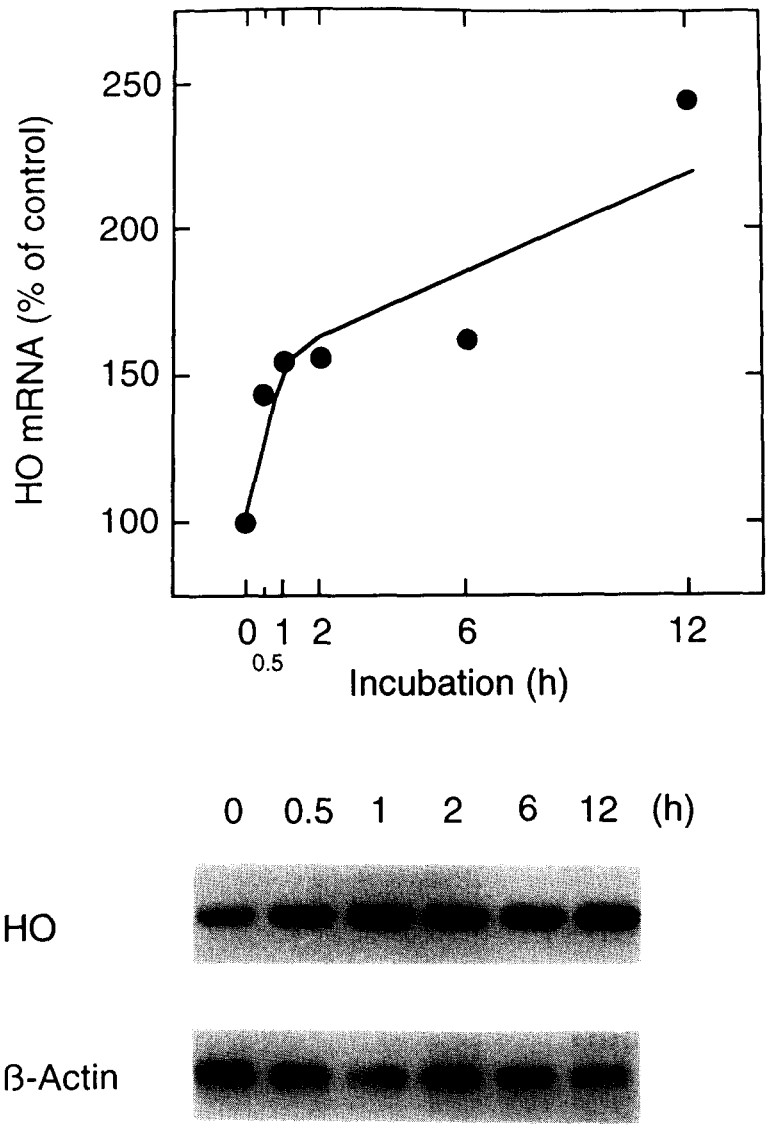

Fig. 5. Effect of IL-6 on the expression of heme oxygenase mRNA. HepG2 cells were incubated in the presence of IL-6 (100 U/mL). Total RNA was isolated and examined by northern blot analysis using the probe for heme oxygenase, as described in Materials and Methods. The level of β-actin mRNA was also examined as a control. Data are expressed as percent of the value of the untreated control.

of SnMP partially restored the IL-6-mediated suppression (58.3% of the 3MC control) (Fig. 6). Similar results were also observed when IL-6 was added together with 3MC and SnMP, i.e. 79.7 and 42.9%, for IL-6/SnMP treatment, and for IL-6 treatment, respectively. Treatment with SnMP alone showed little effect on the level of CYPIA1 mRNA. These findings also confirm that the suppressive effect of IL-6 on CYPIA mRNA is mediated by the induction of heme oxygenase.

DISCUSSION

Depression of cytochrome P450-dependent drug metabolism has been described as an accompaniment to various inflammatory processes in animals [26–28]. Similar changes can be reproduced by treatment of animals with inflammatory cytokines such as IL-1 [29–31], tumor necrosis factor [30, 32], IFN [33, 34], and IL-6 [30, 31]. Our results in this study demonstrated that IL-6 markedly suppresses the expression of 3MC-induced CYPIA1 mRNA and its protein in HepG2 cells (Figs. 1–3). Since our earlier studies using reverse-transcription polymerase chain reaction assays demonstrated that uninduced levels of CYPIA1 mRNA in HepG2 cells were also suppressed by IL-6 treatment [8], these findings indicate that both uninduced and the 3MC-induced CYPIA1 mRNA levels are subject to IL-6-mediated suppression. Thus, IL-6 appears to play an important

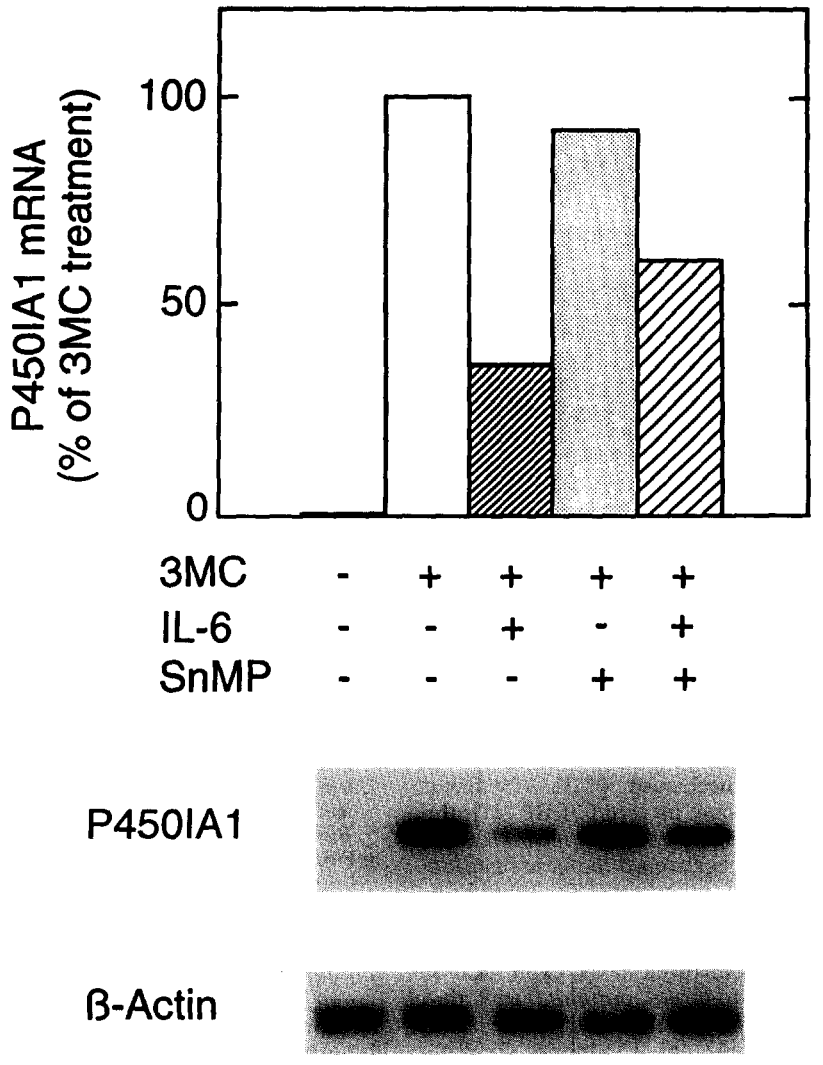

Fig. 6. Effect of Sn-mesoporphyrin on the IL-6-mediated suppression of CYPIA1 mRNA. HepG2 cells were incubated in the presence or absence of 3MC (6 μM), IL-6 (100 U/mL), and SnMP (100 μM). IL-6 and SnMP were added 2 hr prior to 3MC treatment. Cells were harvested 48 hr after the addition of 3MC, and total RNA was isolated and examined by northern blot analysis using the probe for CYPIA1, as described in Materials and Methods. The level of β-actin mRNA was also examined as a control. Data are expressed as percent of the value of the 3MC-treated culture.

role, not only as an inducer of the acute-phase reaction, but also as a mediator that modulates CYP synthesis.

The suppressive effect of IL-6 on CYPIA1 appears to be mediated largely by its transcriptional effect, as judged by nuclear runoff analysis. It is known that suppression of CYPIIC11 mRNA in rats by inflammation also occurs by a transcriptional mechanism [35], whereas that of CYPIIC12 appears to occur at a post-transcriptional level [35]. Other cytokines involved in inflammatory responses, such as IL-1β and IFN, appear to suppress CYP gene expression largely by a transcriptional mechanism [36], although other additional mechanisms such as free radical-mediated degradation may be involved in the case of the action of IFN [34]. Thus, it is possible that there may be yet an additional mechanism(s) for the regulation of IL-6-mediated CYPIA1 gene expression.

The down-regulation of the human CYPIA1 gene by IL-6 appears, at least in part, to involve a decrease in the level of "free heme" [37], resulting from the induction of heme oxygenase. Free heme can be considered as (i) heme that is either synthesized, or taken up recently by cells, but not yet bound as a prosthetic group, or (ii) heme that has just been released from hemeproteins [37]. There are several reasons which indicate that regulation of CYPIA1 may be achieved by free heme. Namely, (i) treatment of cells with IL-6 significantly increased the level of heme oxygenase mRNA (Fig. 5), (ii) inhibition of heme synthesis in the rat is known to suppress the

expression of P450s, while the addition of hemin rescues the inhibition [38, 39], (iii) the concentration–response relationships of IL-6 for CYPIA1 mRNA suppression (Fig. 2) and heme oxygenase induction [23] are very similar, and (iv) the addition of SnMP, a specific inhibitor of heme oxygenase activity (K_i = 0.014 μM [25]), partially restored the IL-6-mediated suppression of CYPIA1 (Fig. 6). Although it is not possible to directly determine the free heme concentration, since it is below the detection level [37], the above findings are all compatible with the assumed changes in the free heme pool.

The suppressive effect of IL-6 on 3MC-induced CYPIA1 concentration was increased markedly by preincubation of cells with IL-6 for 2 hr (Fig. 1), suggesting that there is a deficiency in free heme concentrations. In contrast to pretreatment for 2 hr, a longer preincubation, i.e. 6 hr and 12 hr, had little effect on CYPIA1 mRNA (Fig. 1). Since IL-6 has been shown to increase the activity of ALA synthase, the rate-limiting enzyme of heme biosynthesis, in HepG2 cells [40], it is possible that longer pre-incubation with IL-6 may induce ALA synthase, which may restore heme deficiency. Time courses of induction of ALA synthase and heme oxygenase mRNAs appear to conform to this hypothesis, i.e. heme oxygenase mRNA induction occurred more rapidly than the increase of ALA synthase mRNA (data not shown).

Another possible mechanism of IL-6-mediated CYPIA1 suppression may involve C/EBP [41], the liver-specific DNA binding protein which is known to regulate genes for negative acute-phase reactants such as albumin and α_1-antitrypsin [42]. It has been shown that the structure of C/EBP is homologous to that of IL-6DBP, i.e. NF-IL6 [41, 43], the *trans*-acting factor which is involved in the expression of certain positive acute-phase proteins, and that C/EBP, IL-6DBP and NF-IL6 bind to the same nucleotide sequences [42, 44]. The human CYPIA1 gene is known to contain a sequence, TTTCTGCAATG (−1536 to −1526) [45], which is similar to the consensus sequence recognized by C/EBP, IL-6DBP, and NF-IL6. Thus, it is possible that down-regulation of the human CYPIA1 gene by IL-6 may be mediated by C/EBP. Further studies are needed to define the exact mechanism by which IL-6 suppresses the expression of the human CYP genes.

Our findings in this study define for the first time a role for IL-6 in regulating heme metabolism. These data also suggest that other physiological as well as environmental stimuli, such as heat-shock [15, 46, 47], heavy metals [48, 49], ultraviolet light [50] and oxidant stress [50], which are all known to increase heme oxygenase activity, may influence cytochrome P450 gene expression, and thereby adversely affect drug metabolism. Recent evidence suggests that cytochrome P450 gene expression may also be involved in the elimination of bilirubin [51]. Gene activation of heme oxygenase may be a common link among these changes, and studies concerning this question are currently underway in our laboratory.

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