The role of inorganic metals and metalloporphyrins in the induction of haem oxygenase and heat-shock protein 70 in human hepatoma cells

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The role of inorganic metals and metalloporphyrins in the induction of mRNAs for haem oxygenase and heat-shock protein 70 (hsp70), the two heat-shock proteins, was examined in human HepG2 and Hep3B hepatoma cells. $SnCl_2$, but not Sn–protoporphyrin, was found to be a potent inducer of both haem oxygenase and hsp70 mRNAs. In contrast, $CoCl_2$, $ZnCl_2$ and $FeCl_2$ caused little induction of haem oxygenase and hsp70 mRNAs, whereas the porphyrin complexes of these metals strongly induced haem oxygenase mRNA, without influencing the level of hsp70 mRNA. The induction process was largely transcriptional, as judged by the inhibition of induction by actinomycin D, but not by cycloheximide, and by increased transcription demonstrated by nuclear run-off analysis. Since $CoCl_2$ is a potent inducer of haem oxygenase *in vivo* in animals, the possibility of the biosynthesis of Co–protoporphyrin was examined in human hepatoma cells by incubating them with $CoCl_2$ and protoporphyrin, or δ-aminolaevulinate (ALA), the precursor of protoporphyrin. Both types of treatment led to a potent induction of haem oxygenase mRNA. Co–protoporphyrin formation was also spectrally demonstrated in cells incubated with the metal and ALA. The results of this study indicate that certain metals, e.g. $SnCl_2$, may directly induce haem oxygenase mRNA, whereas with other elements, incorporation of the metal into the porphyrin macrocycle is necessary for induction. Therefore $CoCl_2$, like haemin, may activate the haem oxygenase gene via a haem-responsive transcription factor, whereas $SnCl_2$ may exert its effect via a metal-responsive transcription factor.

INTRODUCTION

Microsomal haem oxygenase (EC 1.14.99.3) catalyses the oxidative cleavage of haem to yield biliverdin IXα [1,2]. Biliverdin IXα is then converted into bilirubin IXα by cytosolic biliverdin reductase. In this series of reactions, haem oxygenase is the rate-limiting enzyme [1–3]. The enzyme is known to be inducible *in vivo* in animals and *in vitro* in cell cultures by its substrate, haemin, and by various non-haem substances, including certain transition elements and heavy metals [2,4–12]. The mechanism by which haem oxygenase is induced by metals is, however, not well understood. In addition, it appears that there are significant differences in the induction response of haem oxygenase between animal and human cells to certain stimuli. For example, haem oxygenase is a heat-shock protein in all rodent cells [13], but it is not usually induced by heat shock in human cells [14] with some exceptions [9,15]. Thus the induction response of human cells to metals is best studied by using isolated human cells in which haem oxygenase induction responses can be demonstrated by metal treatment.

Human HepG2 and Hep3B hepatoma cells, isolated by Knowles et al. [16] from human hepatoblastoma biopsies, retain many properties characteristic of normal hepatic parenchymal cells [16–18]. Unlike most liver cell cultures, HepG2 cells contain significant activities of haem pathway enzymes [19,20] and microsomal cytochrome *P*-450 [21], and they also show an induction response of δ-aminolaevulinate (ALA) synthase [20], ALA dehydratase [19] and haem oxygenase [22] after appropriate chemical treatments.

In this study, we examined the effects of metal ions and their porphyrin chelates on the levels of mRNAs encoding haem oxygenase and heat-shock protein 70 (hsp70) in HepG2 and Hep3B cells. We found that, whereas $CoCl_2$ has little effect, Co–protoporphyrin induces haem oxygenase mRNA at the transcriptional level in human hepatoma cultures [22]. In contrast, $SnCl_2$ acts as an inducer, whereas Sn–protoporphyrin is inactive. Our results also show the biosynthesis of Co–protoporphyrin in human hepatoma cells from the metal and protoporphyrin, or its precursor, and that, with respect to $CoCl_2$ and in contrast with $SnCl_2$, the intracellularly formed metalloporphyrin is the proximate inducer of haem oxygenase mRNA.

MATERIALS AND METHODS

Cell cultures and treatments

HepG2 cells were kindly provided by Dr. Barbara B. Knowles, The Wistar Institute, Philadelphia, PA, U.S.A. Hep3B cells were obtained from American Type Culture Collection, Rockville, MD, U.S.A. Both hepatoma cells were grown in 150 mm × 20 mm tissue-culture dishes (Corning) in minimum essential medium with Earle's salts supplemented with 10% (v/v) defined bovine calf serum (HyClone Laboratories, Logan, UT, U.S.A.), 100 units of penicillin/ml, 100 μg of streptomycin/ml and 2 mM glutamine. Cells were seeded in culture dishes at a confluence of 12.5% in comparison with a parent culture, followed by medium replenishment after 4 days of incubation, and treatments of cultures with chemicals were made 24 h after the medium replenishment. To treat cells with chemicals, the growth medium was removed and saved, cell cultures were replenished with Earle's buffer solution containing chemicals, and incubated for 30 min, unless otherwise stated. After treatment with chemicals, Earle's buffer was replaced with the growth medium and incubation was continued for 3 h before the isolation of total RNA. In experiments in which intracellular formation of metalloporphyrins was examined, cells were incubated with growth medium containing metal salts, and protoporphyrin IX, or its precursor, ALA. Since protoporphyrin was toxic to cells, incubation with protoporphyrin was made for 30 min, but in-

Abbreviations used: ALA, δ-aminolaevulinate; HSE, heat-shock element; hsp, heat-shock protein.

cubation with ALA was made for 24 h, as a nearly maximal amount of protoporphyrin was found in the cell 24 h after incubation. After incubation, cultures were rinsed once with Earle's buffer devoid of Ca^{2+} and Mg^{2+}, and recovered by treatment with 0.25% trypsin containing 0.53 mM EDTA.

Chemicals

Metals were all chloride salts of analytical grade obtained from Fisher Scientific Co. (Fair Lawn, NJ, U.S.A.) or Sigma Chemical Co. (St. Louis, MO, U.S.A.). Porphyrins (free and metal chelates) and ALA were purchased from Porphyrin Products (Logan, UT, U.S.A.). Succinylacetone (4,6-dioxoheptanoic acid) and actinomycin D were purchased from Calbiochem (San Diego, CA, U.S.A.). Other chemicals were obtained from Sigma and were of analytical grade. CaMgEDTA [23] was prepared as follows: for 300 ml of 0.1 M solution, an equimolar solution of EDTA and a solution of $CaCl_2$ and $MgCl_2$ were separately prepared. The Ca/Mg solution was added to the EDTA solution slowly by maintaining the pH of the mixture between 6 and 9 with NaOH. The final pH of the solution was adjusted to 7.4, and the volume of the solution was made up to 300 ml with distilled water. The mixture was then filtered through a Millipore filter (0.22 μm pore size) for sterilization.

Northern-blot analysis

Total RNA (15 μg) was applied to 1.2% (w/v) agarose/formaldehyde gels [24], electrophoresed and transferred to a sheet of Zeta-probe blotting membrane (Bio-Rad, Richmond, CA, U.S.A.). Hybridization was carried out with appropriate RNA probes, followed by RNAase treatment and washing under stringent conditions. Levels of mRNAs were quantified by densitometry using an LKB Ultrascan XL laser densitometer. Experiments were performed in duplicate. Differences between duplicate determinations were within 7%. Results were expressed as the ratio of the amount of the appropriate mRNA in the treated cells to that in the untreated control.

Nuclear run-off transcription assay

Nuclei were isolated from 5×10^7 cells which had been harvested by scraping them off culture dishes using a 'rubber policeman', and washed once in Earle's buffer devoid of Ca^{2+} and Mg^{2+}. Run-off transcription assays were carried out using nuclear lysates in the presence of 200 μCi of [α-^{32}P]UTP (1 Ci = 37 GBq; New England Nuclear) as described previously [25].

cDNA probes

cDNA probes used were human haem oxygenase (pHHO1) [12] and human hsp70 (pH 2.3) [26]. Each cDNA was inserted into a pGEM4z vector (Promega, Madison, WI, U.S.A.) and was transcribed to obtain an RNA probe, by the method of Melton et al. [27].

Determination of Co–protoporphyrin

A modification of the method described by Sinclair et al. [28] was used. After chemical treatment, cells were rinsed with Earle's buffer devoid of Ca^{2+} and Mg^{2+}. Cells that had been removed from a 15 cm culture dish by trypsin treatment were resuspended in 0.5 ml of medium containing 10% (v/v) defined bovine calf serum and 12 mg of BSA. Free porphyrins and haem were extracted into 3.5 ml of a mixture of acetone/conc. HCl (20:1, v/v). To the pellet, 0.5 ml of a mixture of acetic acid/pyridine (10:1, v/v) was added, and after vortex-mixing, 2 ml of chloroform was added, and mixed well. The mixture was centrifuged at 1000 *g* (2600 rev./min), and the spectrum of a supernatant was determined against solvent mixture by scanning from 370 nm to 600 nm using a Cary 118 spectrophotometer.

RESULTS

Effects of $SnCl_2$ on the induction of haem oxygenase mRNA and hsp70 mRNA

Haem oxygenase has been shown to be a stress protein in human cells which can be induced by metals [29], heat shock [9,15] and oxidants [9]. Therefore we examined the effects of inorganic metals and porphyrins to determine whether these elements induce mRNAs for haem oxygenase and hsp70 in Hep3B and HepG2 cells. Among the four metal ions examined, i.e. $CoCl_2$, $ZnCl_2$, $SnCl_2$ and $FeCl_2$, only $SnCl_2$ was found to markedly

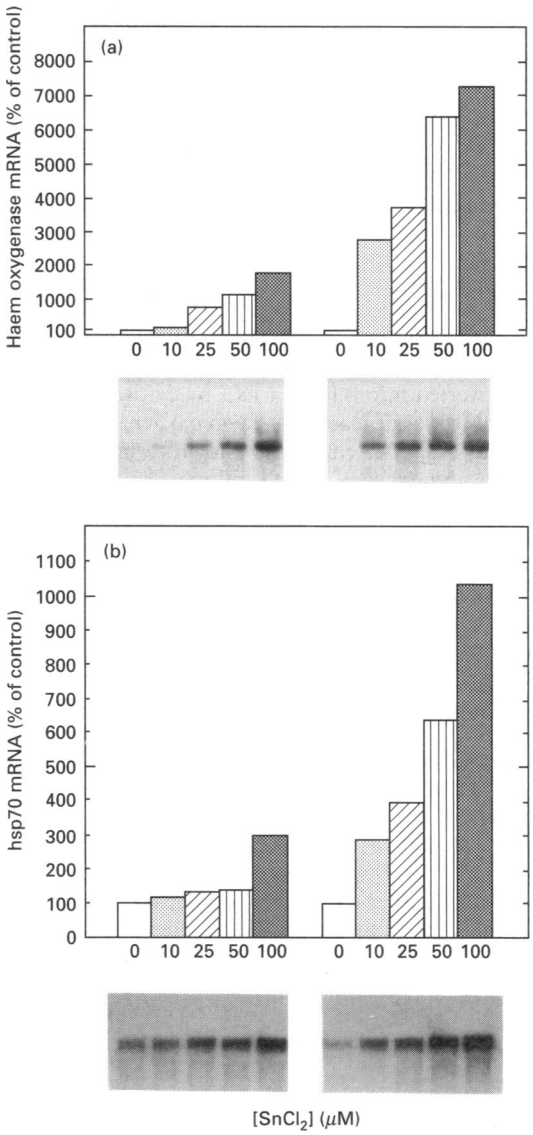

Figure 1 Effects of $SnCl_2$ on the levels of mRNAs encoding haem oxygenase and hsp70 in human hepatoma cells

Cells were incubated with various concentrations of $SnCl_2$ for 30 min. mRNA concentrations were determined from Northern blots by densitometry, as described in the Materials and methods section. (**a**) Haem oxygenase mRNA; (**b**) hsp70 mRNA. The five lanes on the left represent HepG2 cells and the five lanes on the right Hep3B cells.

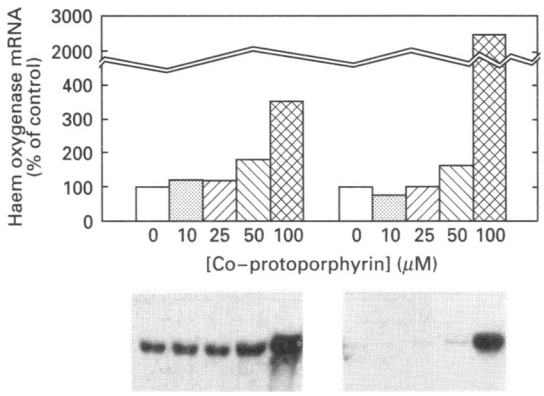

Figure 2 Effects of Co–protoporphyrin on the level of haem oxygenase mRNA in human hepatoma cells

Cells were incubated with various concentrations of Co–protoporphyrin for 30 min. mRNA concentrations were determined from Northern blots by densitometry, as described in the Materials and methods section. The five lanes on the left represent HepG2 cells and the five lanes on the right Hep3B cells.

increase mRNAs encoding haem oxygenase and hsp70 both in HepG2 and Hep3B cells (Figure 1a and 1b). In contrast, $CoCl_2$, $ZnCl_2$, $FeCl_2$, protoporphyrin and mesoporphyrin had little effect on these mRNA levels (results not shown). Induction of both haem oxygenase mRNA and hsp70 mRNA by $SnCl_2$ was clearly dependent on the metal concentration (Figure 1a and 1b).

At the lowest concentration of the metal examined, i.e. 10 μM, the levels of haem oxygenase mRNA were 2-fold and 27-fold greater than the levels in untreated controls in HepG2 and Hep3B cells respectively (Figure 1a). Induction of haem oxygenase mRNA by 18-fold and 73-fold was observed in HepG2 and Hep3B cells respectively at a 100 μM concentration of the metal. The maximal levels of hsp70 mRNA observed at a $SnCl_2$ concentration of 100 μM were 3-fold and 10-fold greater than those in untreated controls, in HepG2 and Hep3B cells respectively (Figure 1b). Although increases in haem oxygenase and hsp70 mRNAs after metal treatment were greater in Hep3B cells than in HepG2 cells, the trend was similar in both cell lines (Figure 1a and 1b).

Effects of $SnCl_2$ and $SnCl_4$ on the induction of haem oxygenase mRNA

We also examined whether the induction response of haem oxygenase mRNA was related to the valence of the metal. Treatment of Hep3B cells with $SnCl_2$ caused a marked induction of haem oxygenase mRNA (Figure 1a), but treatment with $SnCl_4$ did not influence haem oxygenase mRNA levels at all (results not shown). Thus the induction of haem oxygenase mRNA was associated with the lower oxidation state of the metal.

Effects of metalloprotoporphyrins on the induction of haem oxygenase and hsp70 mRNAs

Since haemin is known to be a potent inducer of haem oxygenase both in whole animals [30] and in cultured cells [22,31], we

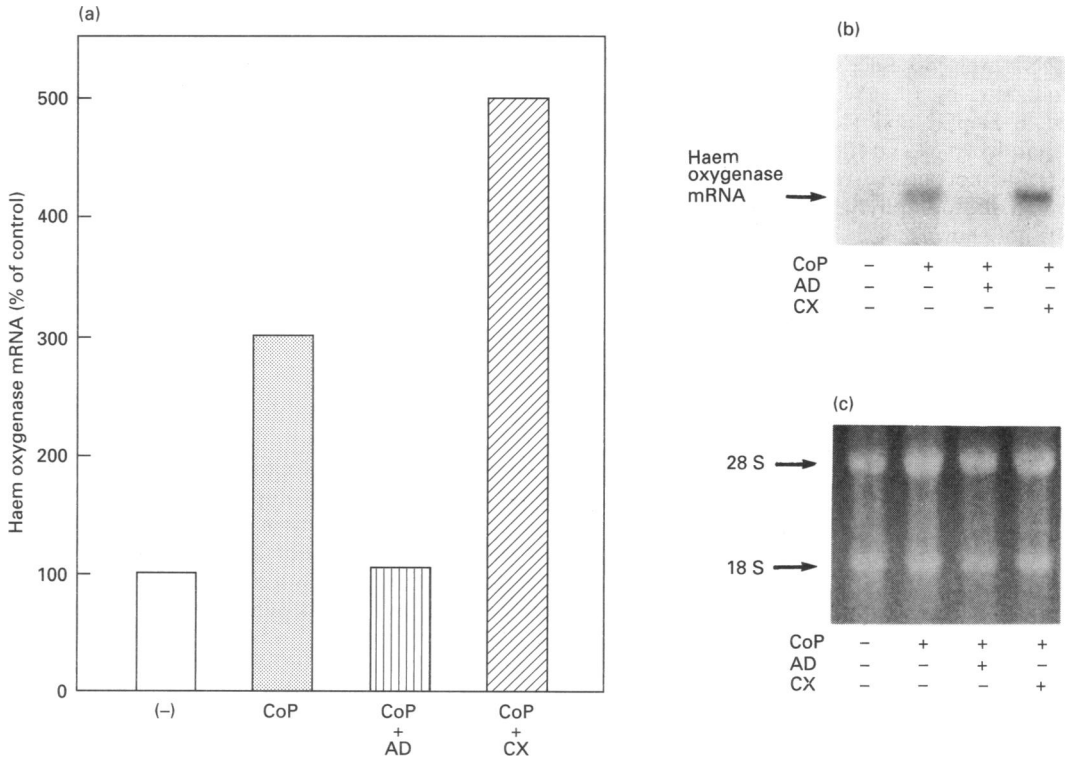

Figure 3 Effects of actinomycin D and cycloheximide on the level of haem oxygenase mRNA in human hepatoma cells

Cells were incubated with 100 μM Co–protoporphyrin (CoP) with actinomycin D (AD) (0.5 μg/ml) or cycloheximide (CX) (2.0 μg/ml) for 30 min. mRNA concentrations were determined from Northern blots by densitometry, as described in the Materials and methods section. (**a**) Levels of haem oxygenase mRNA quantified by Northern-blot analysis; (**b**) autoradiogram of Northern-blot analysis; (**c**) ethidium bromide staining of agarose-gel electrophoresis, showing comparable levels of total RNA loaded in each sample.

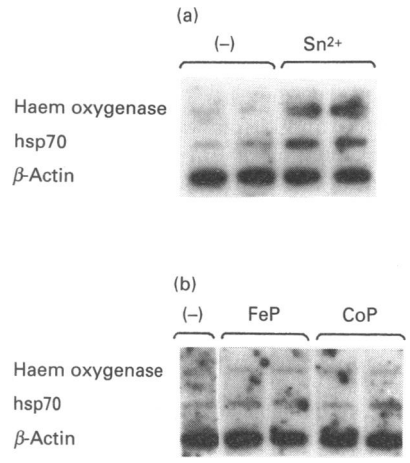

Figure 4 Nuclear run-off transcription assay

Nuclear run-off analysis was performed as described in the Materials and methods section. (**a**) Effect of $SnCl_2$ on the transcription of haem oxygenase, hsp70 and β-actin mRNAs. The left two lanes represent the untreated control, and the right two lanes represent the Sn^{2+}-treated samples. (**b**) Effect of haemin (FeP) and Co–protoporphyrin (CoP) on the transcription of haem oxygenase, hsp70 and β-actin mRNAs. (–), untreated control; FeP, haemin treatment (duplicates); CoP, CoP treatment (duplicates).

examined the effect of protoporphyrin chelates of $CoCl_2$, $ZnCl_2$ and $SnCl_2$ on the induction of mRNAs for haem oxygenase and hsp70. Although $CoCl_2$ and free protoporphyrin (both at 100 μM) had little effect on the level of haem oxygenase mRNA (results not shown), treatment of cells with Co–protoporphyrin resulted in significant induction of haem oxygenase mRNA (Figure 2). The effects of Co–protoporphyrin on the induction of haem oxygenase mRNA were dose-dependent both in HepG2 and Hep3B cells. Induction by 100 μM metalloporphyrin was 3.5-fold and 27-fold in HepG2 and Hep3B cells respectively (Figure 2). Sn–protoporphyrin had no effect on the level of haem oxygenase mRNA (results not shown). Metalloporphyrins such as Fe–, Co–, Zn– and Sn–protoporphyrin had no effect on hsp70 mRNA levels (results not shown).

Transcriptional induction of haem oxygenase by Co–protoporphyrin, Fe–protoporphyrin and $SnCl_2$

Induction of haem oxygenase mRNA appears to be due to transcriptional activation of its gene, since simultaneous treatment of cells with actinomycin D completely abrogated the induction, whereas cycloheximide had no effect (Figure 3a and 3b). The effect of actinomycin D was not due to a difference in the amount of RNA examined, since the amount was comparable for each sample (Figure 3c). Transcriptional effects of Co–protoporphyrin as well as haemin and $SnCl_2$ were also directly demonstrated by nuclear run-off analysis (Figure 4a and 4b). Whereas $SnCl_2$ increased transcription of haem oxygenase and hsp70 genes, haemin and Co–protoporphyrin increased transcription of the haem oxygenase gene but not that of hsp70.

Effects of metallomesoporphyrins on the induction of mRNAs encoding haem oxygenase and hsp70

Mesoporphyrin is a derivative of protoporphyrin in which the vinyl groups have been replaced with ethyl groups. Metal–mesoporphyrin complexes are generally more water-soluble than

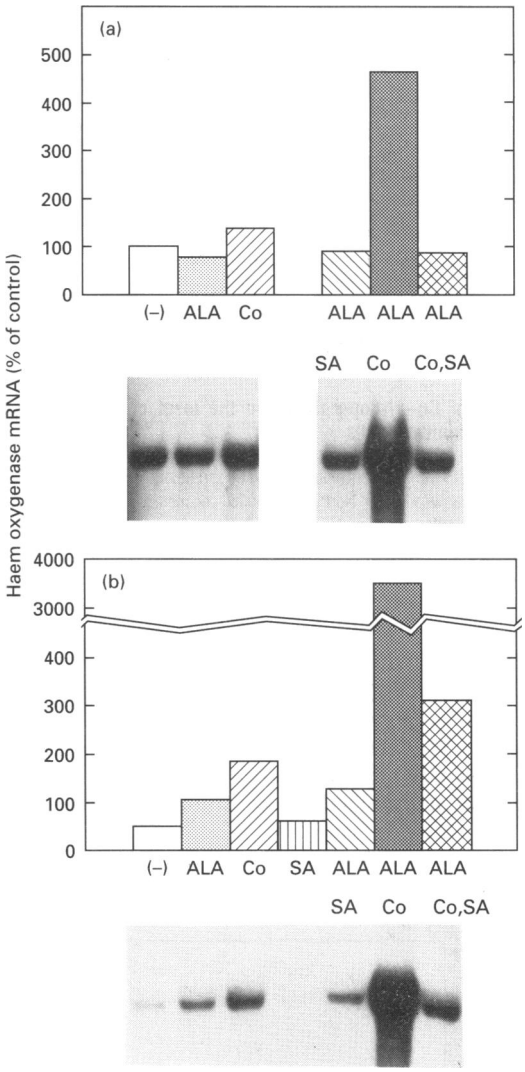

Figure 5 Effects of ALA, $CoCl_2$ and succinylacetone (SA) on the level of haem oxygenase mRNA

Cells were incubated with the chemicals for 24 h. mRNA concentrations were determined from Northern blots by densitometry, as described in the Materials and methods section. (**a**) HepG2 cells; (**b**) Hep3B cells. ALA, 100 μM δ-aminolaevulinic acid; Co, 100 μM $CoCl_2$; SA, 0.5 mM succinylacetone.

their corresponding protoporphyrin complexes, but share many of the properties of the protoporphyrin chelates [32]. As expected, the effects of mesoporphyrin IX chelates of Zn, Co and Sn were generally similar to those of their protoporphyrin chelates (results not shown). For example, we found that Zn–mesoporphyrin and Co–mesoporphyrin induced haem oxygenase mRNA by 4–5-fold in HepG2 cells and by 7–45-fold in Hep3B cells, whereas both metalloporphyrins had little or only a minor effect on hsp70 mRNA ($\leq$ 2 fold) in either cell line (results not shown). Fe–mesoporphyrin had little effect on haem oxygenase mRNA in HepG2 cells, whereas it increased haem oxygenase mRNA in Hep3B cells by 6.7-fold. Fe–mesoporphyrin had no effect on hsp70 mRNA in both cell lines. Sn–mesoporphyrin had essentially no effect on either haem oxygenase or hsp70 mRNA, as was the case with Sn–protoporphyrin. Thus the effects of metal–mesoporphyrin complexes were generally similar to those of the comparable metal–protoporphyrin complexes.

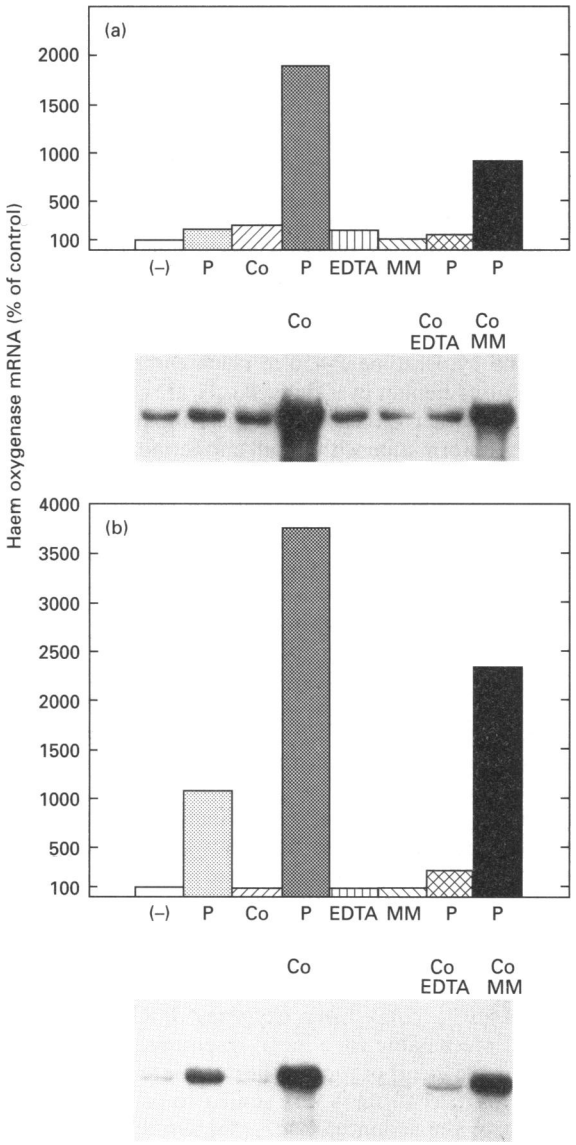

Figure 6 Effects of protoporphyrin, $CoCl_2$, CaMgEDTA and *N*-methylmesoporphyrin on the level of haem oxygenase mRNA

Cells were incubated with the chemicals for 30 min. mRNA concentrations were determined from Northern blots by densitometry, as described in the Materials and methods section. (**a**) HepG2 cells; (**b**) Hep3B cells. P, 100 μM protoporphyrin; Co, 100 μM $CoCl_2$; EDTA, 10 mM CaMgEDTA; MM, 0.35 μM *N*-methylmesoporphyrin.

Intracellular formation of Co–protoporphyrin in the induction of haem oxygenase mRNA

In order to explore the possibility that Co–protoporphyrin could be formed in human hepatoma cells, the cultures were treated with the inorganic metal and ALA, the precursor of protoporphyrin. Cells treated with ALA and $CoCl_2$ for 24 h showed a marked induction of haem oxygenase mRNA (a 4.7- and a 37-fold increase in HepG2 and Hep3B cells respectively) (Figure 5a and 5b). Neither $CoCl_2$ nor ALA alone had a significant effect on the level of haem oxygenase mRNA in HepG2 and Hep3B cells. Cells were also treated with succinylacetone which prevents the formation of protoporphyrin from ALA by inhibiting ALA dehydratase and thereby also the formation of the metalloprotoporphyrin complex. Treatment of cells with succinylacetone greatly decreased the induction of haem oxygenase mRNA elicited by the combined treatment with $CoCl_2$ and ALA. None of these treatments caused significant changes in hsp70 mRNA levels (results not shown). These findings suggest that Co–protoporphyrin is formed in cells incubated with the metal and the protoporphyrin precursor, and that the blockade of protoporphyrin formation (and therefore of Co–protoporphyrin formation also) from ALA by succinylacetone prevented the induction response.

Role of ferrochelatase in the formation of Co–protoporphyrin

To examine the possibility that the intracellular formation of Co–protoporphyrin is dependent on ferrochelatase activity, hepatoma cells were treated with $CoCl_2$ and protoporphyrin, the two substrates for ferrochelatase. Combined treatment with $CoCl_2$ and protoporphyrin caused a marked induction of haem oxygenase mRNA in both cell lines (19- and 38-fold induction in HepG2 and Hep3B cells respectively) (Figure 6). Neither $CoCl_2$ nor protoporphyrin alone induced haem oxygenase mRNA in HepG2 cells. Although some induction of haem oxygenase mRNA (approx. 10-fold) was observed in Hep3B cells by protoporphyrin alone, the induction after the combined treatment with protoporphyrin and $CoCl_2$ was significantly greater (approx. 35-fold compared with the untreated control) than that with protoporphyrin alone.

The effects of two chemicals that interfere with ferrochelatase activity, i.e. CaMgEDTA [23,33] and *N*-methylmesoporphyrin [34], were also examined. When CaMgEDTA was added to the cultures, the induction of haem oxygenase mRNA by $CoCl_2$ and protoporphyrin was abolished in both cell types (Figure 6a and 6b). Similar findings were obtained by treatment of the cultures with *N*-methylmesoporphyrin, although the effect of *N*-methylmesoporphyrin was less pronounced than that of CaMgEDTA (Figure 6a and 6b). Treatment with an inhibitor alone had no significant effect on haem oxygenase mRNA (Figure 6a and 6b).

Spectral evidence for the formation of Co–protoporphyrin

Direct evidence for the synthesis of Co–protoporphyrin in cells incubated with the metal and ALA was obtained by spectrophotometry. There was no detectable Co–protoporphyrin in the extracted protein pellet from untreated control cells, whereas the pyridine haemochrome of the protein pellet from cells treated with both ALA and $CoCl_2$ clearly showed the presence of Co–protoporphyrin in both HepG2 and Hep3B cells (results not shown). The increase in haem oxygenase mRNA caused by intracellularly formed Co–protoporphyrin was significantly greater in Hep3B cells than in HepG2 cells (Figures 5 and 6); however, the amount of Co–protoporphyrin formed was similar in both cell lines. These findings suggest that Hep3B cells are more susceptible to the inducing action of the metalloporphyrin, as was observed with other inducers.

DISCUSSION

It has been shown in animal cells that the activation of the haem oxygenase gene is largely regulated at the transcriptional level [13,35,36]. For example, it has been suggested, by polyribosome-directed cell-free synthesis of haem oxygenase in rat liver [37] and pig alveolar macrophages [38], that there is an increase in the concentration of functional haem oxygenase mRNA after haemin treatment. Haemin-mediated induction of haem oxygenase mRNA in rat C6 glioma cells is also known to be abrogated by simultaneous treatment with actinomycin D [13]. Transcriptional

stimulation of the haem oxygenase gene has also been demonstrated by nuclear run-off transcription assays in mouse Hepa cells treated with haemin or $CdCl_2$ [36]. Our findings in this study on the induction of haem oxygenase mRNA in human hepatoma cells after various treatments are also consistent with these earlier data, and indicate that gene activation of haem oxygenase in human hepatoma cells is also under a transcriptional control.

Treatment of human hepatoma cells with $SnCl_2$ strongly induced haem oxygenase mRNA as well as hsp70 mRNA. This effect was dependent on the valence of the metal ion, since $SnCl_2$ was a potent inducer, whereas $SnCl_4$ was inactive. A similar finding was earlier reported for arsenite- [39] and antimony- [40] mediated induction of hepatic haem oxygenase in the rat. It is also known that erythrocyte ALA dehydratase activity is inhibited in mice treated with $SnCl_2$, but not with $SnCl_4$ [41]. Since haem oxygenase has been shown to be a stress-inducible protein in both animal [29] and human cells [9–11], the induction of haem oxygenase mRNA by $SnCl_2$, but not by $SnCl_4$, suggests that the lower oxidation state of the inorganic metal may be more stressful to the cell.

$SnCl_2$ is similar to Cd^{2+} and As^{3+} [9,10] in that the free metal is capable of inducing mRNAs encoding both haem oxygenase and hsp70, but is different in its action from $CoCl_2$, $ZnCl_2$ or $FeCl_2$ which have little or no effect on these mRNAs. The promoter sequences necessary for heat-shock and metal-ion induction of the human hsp70 gene are closely located in a domain extending from positions −68 to −107 [26]. The sequence starting at position −91 is related to a metal-responsive element in the metallothionein II gene [35,42], and the single heat-shock element (HSE) centred at position −100 has been found to be essential for stress-induced transcription of the human hsp70 gene [43]. It is known that there is also a motif in the rat haem oxygenase gene containing two copies of core sequences of the metal regulatory elements found in the metallothionein gene, and both the rat and the human haem oxygenase genes contain similar HSEs [13]. Thus metal ions such as Sn^{2+}, Cd^{2+} and As^{3+} which can activate the promoter of the hsp70 gene may also activate the promoter of the haem oxygenase gene, via activation of a metal-responsive transcription factor [36].

In contrast with $SnCl_2$, $CoCl_2$ had no significant effect on the induction of haem oxygenase mRNA in human hepatoma cells (results not shown), in rat hepatoma cells [29] and in mouse Hepa cells [36]. The lack of induction of haem oxygenase mRNA by $CoCl_2$ in these cell culture systems differs significantly from the potent inducing action of the metal in whole animals [4]. On the other hand, Co–protoporphyrin is a potent inducer of haem oxygenase mRNA both *in vivo* in rats [44,45] and, as shown in these studies, in cultured human hepatoma cells (Figures 2 and 3). A similar situation was also observed with $FeCl_2$ and $ZnCl_2$, which alone did not induce haem oxygenase mRNA in cultured hepatoma cells, but their corresponding porphyrin chelates were potent inducers of haem oxygenase mRNA.

Haem oxygenase is also a heat-shock protein, and heat treatment activates genes encoding both haem oxygenase and hsp70 in rat cells [13] and in human fibroblasts [9] and Hep3B cells [15], but induction of haem oxygenase mRNA in human hepatoma cells by metalloporphyrins can occur without influencing the level of hsp70 mRNA. Using rat glioma cells, Alam et al. [36] suggested that there are at least two distinct modes of regulation of the rat haem oxygenase gene: induction mediated by a heat-shock transcription factor and induction mediated by a haem-responsive transcription factor. Our findings are consistent with this view in that haemin activates the haem oxygenase gene, but not the hsp70 gene, in human cells. Although it has been reported that haemin was able to induce transcription of the human hsp70 gene in a human erythroleukaemia cell line K562 [46], this effect is probably due to the unique feature of erythroid cells, since haemin fails to induce hsp70 gene expression in other non-erythroid cell lines [46].

The formation of Co–protoporphyrin *in vivo* in animals has been demonstrated by e.p.r. [47] as well as by a spectrophotometric method [48] in livers of rats treated with $CoCl_2$, and by a spectrophotometric method in chicken hepatocyte cultures after treatment with $CoCl_2$ and ALA [28]. The formation of Co–protoporphyrin has been suggested to account for the inhibitory action of $CoCl_2$ on the synthesis of ALA synthase in the rat [49] and cytochrome *P*-450 in chick embryos [50]. Our findings in cultured human liver-derived cells are consistent with those reported in other species, and suggest the possibility that metalloporphyrin formation with cobalt and certain other metals, as with iron, also occurs in human cells.

Studies in chick embryo suggested that ferrochelatase may be involved in the formation of Co–protoporphyrin [28]. Since *N*-alkylated porphyrin is a specific inhibitor of ferrochelatase [51], we used *N*-methylmesoporphyrin in human hepatoma cells and directly demonstrated decreased formation of Co–protoporphyrin by the inhibitor treatment (Figure 6). Inhibition of the ferrochelatase-mediated incorporation of $CoCl_2$ into protoporphyrin by CaMgEDTA, a chemical that chelates iron thereby competing with ferrochelatase activity, was even greater than that brought about by *N*-methylmesoporphyrin, and provides further evidence for the requirement of ferrochelatase in the formation of Co–protoporphyrin. Finally, our data show that the formation of protoporphyrin from ALA is a prerequisite for the formation of Co–protoporphyrin from the inorganic metal, since succinylacetone prevented this process (Figure 5).

Thus our findings in this study demonstrate that there are at least two distinct mechanisms of haem oxygenase mRNA induction by metals in human hepatoma cells. Namely, certain metals such as $SnCl_2$ induce haem oxygenase mRNA as well as hsp70 mRNA, presumably via a metal-responsive factor, which does not require the incorporation of the metal into the porphyrin macrocycle. This mechanism is very similar to or identical with that proposed for the action of $CdCl_2$ [36]. On the other hand, metals such as $CoCl_2$, $ZnCl_2$ and $FeCl_2$ require the biosynthesis of their corresponding porphyrin complexes for induction of haem oxygenase mRNA. Since these biosynthetic haem analogues, like haemin, do not activate the hsp70 gene, it is probable that they induce haem oxygenase via a haem-responsive transcription factor [12], rather than via a metal-responsive transcription factor or HSE [36]. These findings thus point up the differential mechanisms by which metals may induce haem oxygenase and the potential significance of the intracellular formation of metalloporphyrins from certain elements and thus of the importance of ferrochelatase, in the activation of the haem oxygenase gene in human liver cells.

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