J. Biochem. 124, 628-633 (1998)

Involvement of the Tyrosine Phosphorylation Pathway in Induction of Human Heme Oxygenase-1 by Hemin, Sodium Arsenite, and Cadmium Chloride[1]

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Received for publication, April 30, 1998

The effect of a tyrosine kinase inhibitor, herbimycin A, on the induction of heme oxygenase-1 (HO-1) mRNA in HeLa cells upon exposure to hemin, sodium arsenite and cadmium chloride was examined. The induction of HO-1 mRNA by hemin was inhibited when the cells were pretreated with herbimycin A. Herbimycin also inhibited arsenite- and cadmium-dependent induction of HO-1 mRNA in a dose-dependent manner, but less inhibition was observed in cadmium-treated cells than in ones treated with hemin- or arsenite. Genistein (50 μM), another tyrosine kinase inhibitor, also inhibited the induction of HO-1 mRNA by hemin, arsenite, and cadmium. Nuclear runoff assays revealed that herbimycin blocked the hemin-induced transcription of the HO-1 gene. The induction of HO-1 mRNA by hemin in human peripheral blood mononuclear cells was inhibited by herbimycin. The tyrosine phosphorylation of a protein with a molecular mass of 66 kDa in the cells was increased by hemin- or arsenite-treatment, and this increase was inhibited by treatment with 5 μM herbimycin. When HeLa cells were treated with a specific inhibitor of the mitogen-activated protein kinase (MAPK)/extracellular-signal regulated kinase cascade, PD58059 (100 μM), suppression of the cadmium-dependent HO-1 induction was not observed, but the hemin- or arsenite-dependent induction was slightly inhibited. SB203580, an inhibitor of p38 MAPK, did not affect the HO-1 induction. These results indicated that signal transduction involving tyrosine kinase rather than the MAPK family regulates the induction of human HO-1 gene expression by stress inducers.

Key words: arsenite, cadmium, heme oxygenase-1, hemin, tyrosine phosphorylation.

Heme oxygenase [EC 1.14.99.3] catalyzes the initial reaction in heme catabolism: the oxidative cleavage of the α-mesocarbon bridge of heme molecules to yield equimolar quantities of biliverdin IXa, carbon monoxide, and iron. Biliverdin is subsequently converted to bilirubin through the action of biliverdin reductase. Two isozymes of heme oxygenase, heme oxygenase-1 (HO-1) and HO-2, have been identified to date in all animals thus far examined (*1-3*). While HO-2 is constitutively expressed in various tissues, including brain, liver, and testis (*2, 3*), the expression of HO-1 is markedly induced in response to the substrate, heme, and a variety of environmental conditions (heat shock and hypoxia) and agents, including heavy metals, TPA, and UV irradiation (*4, 5*).

Stimulation of HO-1 expression by most inducers is controlled primarily at the level of gene transcription (*5*). A series of reports have described distinct *cis*-acting DNA elements within the human, rat, or mouse HO-1 gene that are involved in induction by cytokines (*6*), heat shock (*7*), TPA (*8*), UV irradiation (*9*), heavy metals (*10, 11*), and heme (*11, 12*). Several transcriptional factors that interact with these *cis*-acting elements have been demonstrated (*7-12*). It is also evident that the induction of HO-1 by TPA, an activator of protein kinase C, is mediated by protein kinase C, probably linked to the phosphorylation of *c-jun* and *c-fos* (*12*). There are several signal transduction pathways involving tyrosine kinase and the MAPK family. The MAPK family includes JNK, p38 MAPK, and ERK, which are activated by cellular stress (*13, 14*). However, the intracellular signals from stress inducers to nuclear factors that activate the HO-1 gene remain to be clarified. In order to delineate intracellular signals that modulate expression of the HO-1 gene, we have investigated possible roles of tyrosine kinases and/or MAPK by using specific inhibitors. Here we report that the induction of human HO-1 with hemin, arsenite, and cadmium in HeLa and peripheral blood mononuclear cells is dependent upon a putative tyrosine kinase that is specifically inhibited by

[1] This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and by a research grant from Sumitomo Chemical Co.

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Abbreviations: HO-1, heme oxygenase-1; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; SDS, sodium dodecylsulfate; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate; ERK, extracellular signal regulated protein kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase/extracellular signal-regulated protein kinase kinase; JNK, c-Jun N-terminal kinase; bp, base pair(s); kDa, kilodaltons.

herbimycin A and genistein. Whereas, inhibitors of the MAPK family did not affect the HO-1 induction. Enhancement of the phosphorylation of a 66-kDa protein in hemin-treated mononuclear cells was inhibited by herbimycin, suggesting that a target for regulation may be intracellular signals.

MATERIALS AND METHODS

Materials—[α-^{32}P]dCTP (6,000 Ci/mmol) and [α-^{32}P]-UTP (800 Ci/mmol) were obtained from NEN. Restriction endonucleases and nucleic acid-modifying enzymes were obtained from Toyobo. Human HO-1 cDNA (pHHO-1) was a kind gift from Dr. S. Shibahara, Tohoku University. Herbimycin A was a product of Wako Pure Chemicals. Genestein, PD58059, and SB203580 were from Calbiochem. Hemin was dissolved in dimethylsulfoxide at a concentration of 20 mM, and then diluted 20-fold with PBS containing 10 mg/ml BSA. All other chemicals used were of analytical grade.

Cell Cultures—Hela cells were maintained in DMEM supplemented with 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin. To expose the cells to hemin, arsenite or cadmium, the cells were washed twice with PBS, placed in DMEM containing 1 mg/ml BSA, and then incubated in the presence of each insulting agent at 37°C for the indicated periods. For treatment with tyrosine kinase inhibitors, the cells were incubated with the inhibitors at 37°C for 1 h prior to the addition of inducers of HO-1. After incubation with the inducers, the cells were washed twice with PBS. Peripheral blood mononuclear cells were isolated from peripheral blood of normal donors using the Ficoll-Hypaque isolation procedure, as described previously (*15*). The cells were cultured in RPMI1640 medium containing 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin and 0.5 μg/ml concanavalin A for 24 h. The cells were harvested, washed twice with PBS, and then incubated in RPMI1640 medium containing 1 mg/ml BSA and 10 μM hemin.

Northern Blots—Total RNAs from cells were prepared by the guanidium isothiocyanate method. The RNA preparations were electrophoresed in a 1% agarose gel under denaturing conditions, and then transferred onto a nylon filter. The conditions for hybridization and washing were as described (*16*).

Nuclear Runoff Transcription Assay—Nuclei from 5×10^7 cells were prepared by cell lysis in a solution of 10 mM Tris-HCl buffer, pH 7.5, 10 mM NaCl, 2 mM $MgCl_2$, and 1% Nonidet P-40, and then resuspended in a nuclear stock solution (40% glycerol, 50 mM Tris-HCl buffer, pH 8.3, 5 mM $MgCl_2$, and 0.1 mM EDTA), and stored at −80°C. The nuclear runoff reaction was performed under conditions similar to those described previously (*17*). Briefly, nuclei were incubated in a final volume of 400 μl of 5 mM Tris-HCl buffer, pH 8.0, containing 2.5 mM $MgCl_2$, 150 mM KCl, 0.25 mM triphosphate of A, G, and C, and 250 μCi of [α-^{32}P]UTP, at 30°C for 30 min. ^{32}P-labeled RNA was isolated and then hybridized at 42°C for 48 h. The filters were washed twice with 2×SSC/0.1% SDS at 65°C for 30 min each, followed by two washes with 0.1×SSC/0.1% SDS.

Immunoblot Analysis—Cells were lysed in Laemmli's sample buffer (*18*). The samples were sonicated and boiled. The cellular proteins were analyzed by SDS-polyacrylamide gel electrophoresis, and then transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed using a monoclonal antibody to phosphotyrosine (4G10) as the primary antibody.

Determination of Heme Associated with Cells—HeLa cells were washed twice with PBS, and then the amount of heme associated with the cells was determined as described previously (*19*). Briefly, the cells were suspended in 2 M oxalate and then heated at 110°C for 30 min to convert heme to protoporphyrin IX. The fluorescence of protoporphyrin was quantitated with a fluorescence spectrophotometer (Hitachi Model MPF 400).

RESULTS

Effects of Tyrosine Kinase Inhibitors on the Induction of HO-1 mRNA—Total RNA was isolated from HeLa cells incubated in the presence of hemin, and then the level of HO-1 mRNA was determined by Northern blot analysis. A 1.5 kbp band corresponding to HO-1 mRNA in HeLa cells treated with 10 μM hemin increased with time up to 90 min. No change in the level of HO-1 mRNA was observed in the cells without hemin. Treatment of the cells with a protein kinase inhibitor, herbimycin A (10 μM), for 1 h prior to the addition of hemin prevented the induction of HO-1 mRNA (Fig. 1). With increasing concentrations of herbimycin A, the HO-1 induction by hemin decreased in a dose-dependent manner (Fig. 2A). Similar inhibition by herbimycin was observed in cells treated with 10 μM sodium arsenite. In 1 μM cadmium chloride-treated cells, HO-1 induction was decreased by herbimycin, but the level of the inhibition was less than that for hemin- or arsenite-treated cells (Fig. 2, B and C). The pretreatment of HeLa cells with 50 μM genistein, another tyrosine kinase inhibitor, also inhibited the inducer-dependent induction of HO-1 mRNA in all three cases. To eliminate the possibility that tyrosine kinase inhibitors inhibit the uptake of exogenously supplied heme by cells, the heme content of hemin-treated HeLa cells with or without herbimycin was determined. About 8% of the added heme was recovered from the cells incubated with 10 μM herbimycin and 10 μM hemin together at 37°C for 30 min, which was similar to

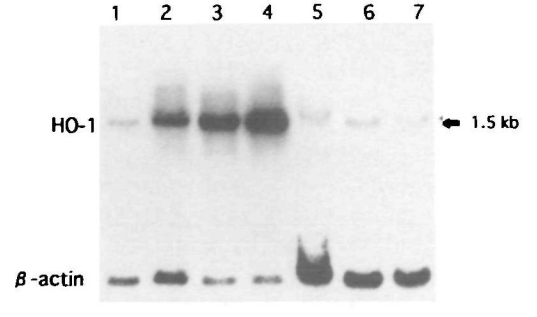
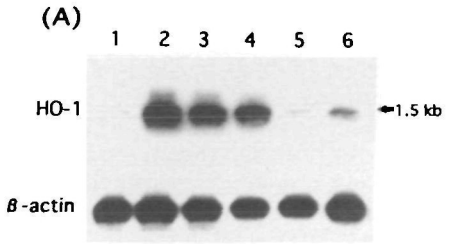
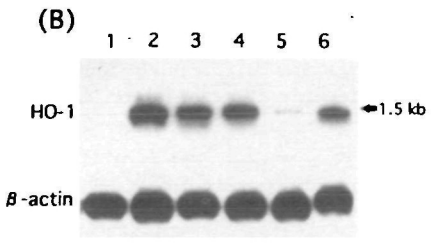


Fig. 1. **Effect of herbimycin A on the induction of HO-1 mRNA in hemin-treated HeLa cells.** Cells were incubated without (lanes 1-4) or with 10 μM herbimycin (lanes 5-7) for 1 h prior to the addition of 10 μM hemin, and were then further incubated for 0 min (lane 1), 30 min (lanes 2 and 5), 60 min (lanes 3 and 6), or 90 min (lanes 4 and 7). Total RNA (10 μg) from the indicated cells was electrophoresed on a formaldehyde/agarose gel and then transferred to a nylon membrane. The filter was hybridized with ^{32}P-labeled cDNA for human HO-1 (upper panel) or β-actin (lower panel).





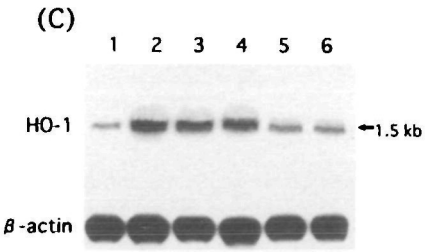


Fig. 2. **Dose-dependent inhibition by tyrosine kinase inhibitors of the HO-1 mRNA induction in HeLa cells.** Cells were untreated (lanes 1 and 2), or treated with 1 μM (lane 3), 5 μM (lane 4) or 10 μM herbimycin (lane 5) or 50 μM genistein (lane 6) for 1 h. The cells were then incubated without inducers (lane 1), or with 10 μM hemin (A), 10 μM sodium arsenite (B), or 1 μM cadmium chloride (C) for 2 h. Equal amounts of total RNA (10 μg) were electrophoresed and analyzed by Northern blotting.

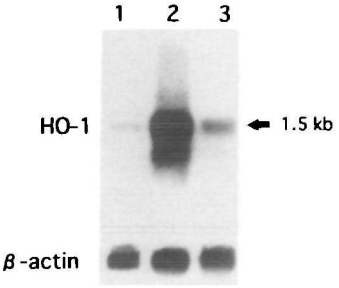


Fig. 3. **Effect of herbimycin on the hemin-dependent induction of HO-1 mRNA in human peripheral blood mononuclear cells.** Cells were untreated (lanes 1 and 2), or treated with 5 μM herbimycin (lane 3) for 1 h, and then incubated without (lane 1) or with 10 μM hemin (lanes 2 and 3) for 90 min. Northern blotting was performed as described.

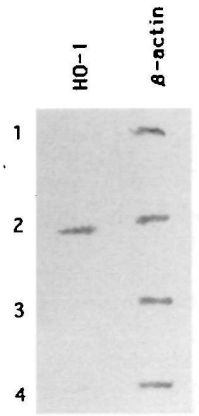


Fig. 4. **Rate of transcription of the HO-1 gene.** Nuclei were isolated from HeLa cells untreated (1) or treated with 20 μM hemin (2), 10 μM herbimycin (3), or 10 μM herbimycin plus 20 μM hemin (4) for 30 min. Runoff transcription assays were performed, and ^{32}P-labeled RNA was hybridized to a nylon membrane which had been blotted with denatured plasmids containing human cDNA for HO-1 or β-actin.

that recovered from the cells incubated with 10 μM hemin (data not shown). We next examined the effect of herbimycin on the HO-1 mRNA induction by hemin in human peripheral blood mononuclear cells. As shown in Fig. 3, herbimycin at 5 μM inhibited the hemin-mediated induction of HO-1 mRNA. These results suggest that the induction of HO-1 expression is regulated through a signal transduction pathway *via* tyrosine kinases.

Effects of Hemin and Herbimycin A on the Rate of Transcription of the Human HO-1 Gene—To determine whether the observed changes in the level of HO-1 mRNA are due to variation in the transcription of the gene or not, the rate of transcription of the HO-1 gene was examined by nuclear runoff analysis. The rate of transcription of the HO-1 gene was up-regulated by hemin treatment (Fig. 4), a finding consistent with observations by Takeda *et al.* (*10*), and Fujita and Sassa (*20*). Treatment of HeLa cells with herbimycin A (10 μM) for 1 h prior to the addition of hemin (20 μM) decreased the hemin-dependent increase in transcription of the gene. The transcription rate of the β-actin gene in HeLa cells was not influenced by any treatment (Fig. 4), indicating that signal transduction of tyrosine phosphorylation regulates the transcription rate of the HO-1 gene.

Effects of MAPK Inhibitors on the Induction of HO-1 mRNA—Recently, it was reported that the MAPK pathways in various cells are involved in the response to environmental stress inducers (*13, 14*). To determine whether or not the signals of the MAPK family are involved in the HO-1 induction by arsenite, cadmium, and hemin, HeLa cells were pretreated for 1 h with PD58059, an inhibitor of MEK. The HO-1 mRNA induction by the three inducers was not suppressed at 25 μM (Fig. 5, lanes 3, 6, and 9). At 100 μM, the arsenite- and hemin-dependent induction was slightly inhibited. When the cells were pretreated with 10 μM SB203580, an inhibitor of p38 MAPK, the HO-1 induction by the three inducers was not suppressed (Fig. 6). These results suggest that ERK or p38 MAPK does not directly contribute to the activation of the HO-1 gene by heavy metals and hemin.

Tyrosine Phosphorylation of Cellular Proteins on Hemin- or Arsenite-Treatment—To examine the tyrosine phosphorylation of cellular proteins in hemin- and arsenite-treated HeLa cells, cellular proteins were analyzed by SDS-polyacrylamide gel electrophoresis, and then immunoblotting was performed with a monoclonal antibody to phosphotyrosine (4G10). As it is known that tyrosine phosphorylation generally occurs in tumor cells because of cell growth, many major phosphotyrosyl proteins were found, and no changes in the phosphoproteins so far examined were observed in HeLa cells upon exposure to hemin, sodium arsenite, or herbimycin for 2 to 60 min

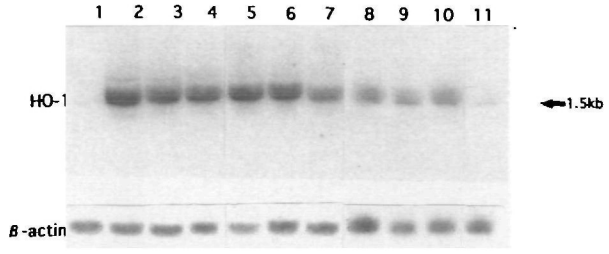


Fig. 5. **Effect of PD58059 on the induction of HO-1 mRNA in hemin-, arsenite-, and cadmium-treated HeLa cells.** The conditions for the treatment of HeLa cells with PD58059 and RNA blotting were similar to those given in the legend to Fig. 2. Cells were untreated (lane 1), or treated with 10 μM hemin (lane 2), 10 μM hemin+25 μM PD58059 (lane 3), 10 μM hemin+100 μM PD58059 (lane 4), 10 μM arsenite (lane 5), 10 μM arsenite+25 μM PD58059 (lane 6), 10 μM arsenite+100 μM PD58059 (lane 7), 2 μM cadmium (lane 8), 2 μM cadmium+25 μM PD58059 (lane 9), 2 μM cadmium+100 μM PD58059 (lane 10), or 100 μM PD58059 (lane 11).

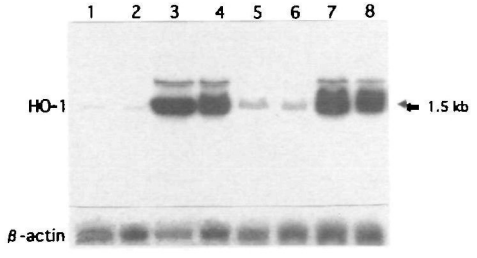


Fig. 6. **Effect of SB203580 on the induction of HO-1 mRNA in HeLa cells.** The conditions for cell treatment with SB203580 and RNA blotting were similar to those given in Fig. 5. The cells were treated without (lane 1), or with 10 μM SB203580 (lane 2), 10 μM hemin (lane 3), 10 μM hemin+10 μM SB203580 (lane 4), 2 μM cadmium (lane 5), 2 μM cadmium+10 μM SB203580 (lane 6), 10 μM arsenite (lane 7), or 10 μM arsenite+10 μM SB203580 (lane 8).

(data not shown). We next examined the tyrosine phosphorylation of cellular proteins in human peripheral blood mononuclear cells since we suspected that a small number of major phosphotyrosyl proteins would be observed in primary isolated cells. When the cells were treated with 20 μM hemin, a phosphoprotein appeared with a molecular mass of 66 kDa was observed within 10 min (Fig. 7), and its level was maintained thereafter up to 60 min. Pretreatment of the cells with herbimycin for 1 h resulted in disappearance of the 66 kDa phosphoprotein induced by hemin. Treatment of the cells with 10 μM sodium arsenite also led to an increase in the 66 kDa phosphoprotein (Fig. 7, lane 6). The increase in the phosphoprotein was decreased by herbimycin A treatment (data not shown). These results indicated that the phosphorylation of the 66 kDa protein in human peripheral blood mononuclear cells increases with concomitant activation of HO-1 gene expression.

DISCUSSION

The present study is the first to demonstrate that tyrosine phosphorylation is involved in the hemin-, arsenite-, and cadmium-dependent induction of HO-1 mRNA in human cells. Pretreatment of HeLa cells with tyrosine kinase inhibitors, herbimycin, and genistein, resulted in the inhibition of the induction of HO-1 mRNA in a dose-dependent

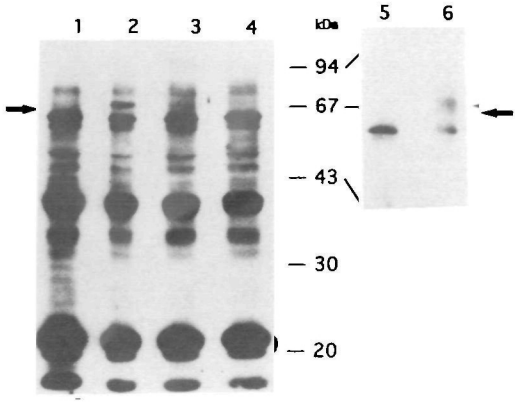


Fig. 7. **Phosphotyrosyl proteins in human peripheral blood mononuclear cells treated with hemin, arsenite, and herbimycin.** Cells were untreated (lanes 1, 2, 5, and 6), or treated with 5 μM herbimycin (lanes 3 and 4) for 1 h, and then further incubated without (lanes 1, 4, and 5), or with 10 μM hemin (lanes 2 and 3) or 10 μM sodium arsenite (lane 6) for 10 min. The cellular proteins were analyzed on an 11% (lanes 1-4) or 7.5% gel (lanes 5 and 6), and then immunoblotting was performed as described. Arrows indicate the position of the 66 kDa protein.

manner. Herbimycin also inhibited the HO-1 mRNA induction in human peripheral blood mononuclear cells. Nuclear runoff assaying of transcription of the HO-1 gene showed that the expression is regulated at the transcriptional level, a finding consistent with the previous observations by Takeda *et al.* (*10*) and Fujita and Sassa (*20*), and the target of tyrosine kinase inhibitors therefore appeared to be a pre-transcriptional event. The inhibition of the HO-1 gene activation by tyrosine kinase inhibitors was not due to a decrease in heme uptake by the cells treated with these chemicals since the cellular levels of heme in hemin- and herbimycin-treated cells were similar to that in hemin-treated cells. In addition to heme- or heavy metal-dependent induction of HO-1, the enzyme is also induced by agents causing oxidative stress, including glutathione depletors, electrophiles, hypoxia, and NO (*21-24*). Furthermore, TPA- (*8*), UV irradiation- (*9*), and heat shock-responsive elements (*25*) in the 5′-promoter region of the human HO-1 gene have been separately characterized. The diversity of HO-1 inducers suggests the presence of various signals for multiple regulatory elements in this gene. Based on the present observation that tyrosine kinase inhibitors inhibit hemin-, cadmium-, and arsenite-dependent induction, which occurs with different regulatory mechanisms, HO-1 gene activation by various inducers may be regulated through the tyrosine kinase-dependent pathway.

The induction of HO-1 mRNA by hemin, arsenite, and cadmium was sensitive to herbimycin, but the drug sensitivity of the cadmium-dependent induction was less than that of the hemin- or arsenite-dependent induction. In this connection, a 20 bp cadmium-responsive element has been found between ~4 and ~4.5 kb upstream from the transcriptional initiation site of the human HO-1 gene (*10*). This element did not respond to the hemin-dependent induction, and the hemin- or arsenite-responsive element(s) in the human HO-1 gene has not been characterized. Furthermore, it is unclear whether or not the mechanism of induction of HO-1 by arsenite is the same as that in the case of hemin. Thus, the different sensitivities of

inducer-dependent HO-1 induction to herbimycin are ascribed to different mechanisms of cadmium- and other inducer-dependent induction of HO-1 mRNA, although the involvement of a tyrosine kinase pathway in the activation of the human HO-1 gene is apparent.

Recent studies (*26, 27*) showed that arsenite activates the MAPK family, including p38 MAPK and ERK, in rat pheochromocytoma PC12 cells, and human epidermoid carcinoma A431 cells and embryonic kidney cells. Furthermore, cadmium led to sustained activation of ERK that was correlated with the induction of *c-fos* in rat mesangial cells (*28*). The present study showed that an MEK inhibitor, PD58059, at high concentrations caused slight suppression of HO-1 induction by arsenite and hemin, but was not effective on cadmium-dependent induction. Furthermore, SB203580 did not suppress HO-1 induction. Thus, different cellular signals depending on the cell species or stimulus appear to be involved in the HO-1 induction, or hemin and arsenite induce HO-1 mRNA at least in part through possible activation of MAPK. We are now further studying the relation of activation of the MAPK family by environmental stress to the induction of stress-induced genes.

Alam *et al.* (*12*) reported that a 161 bp fragment (AB1), ~10 kbp upstream of the transcription start site of the mouse HO-1 gene, functions as a basal level- and inducer-dependent enhancer. All inducers, including hemin, cadmium, H_2O_2, and TPA, require intact AP-1 binding sites for activation of the AB1/chloramphenicol acetyltransferase (CAT) fusion gene, but some of these agents clearly utilize either different AP-1 superfamily proteins and/or different AP-1 transcription activation pathways for this process. Enhancement of the expression of the AB1/CAT fusion gene by TPA was sensitive to protein kinase C inhibitors, which was consistent with the finding that TPA requires activation of protein kinase C, which not only stimulates the expression of *c-fos* and *c-jun* (*29*), but also increases the binding activity of dephosphorylated *c-jun* as to AP-1 binding sites (*30*). In contrast, neither protein kinase C nor tyrosine kinase is involved in the mouse HO-1 induction by H_2O_2 or hemin (*12*). The present study demonstrates the involvement of the tyrosine phosphorylation pathway in the hemin- and arsenite-dependent activation of the human HO-1 gene. The reason for this discrepancy is unknown, but it may reflect the fact that different cell lines were utilized as the human and mouse sources. More importantly, we examined the induction of HO-1 mRNA, while Alam *et al.* examined the enhancement of AB-1/CAT fusion gene expression in stably transfected cells (*12*), finding that the activity of the reporter gene did not always reflect the expression of the HO-1 gene.

We found that a 66 kDa protein in human peripheral blood mononuclear cells was phosphorylated on hemin- and arsenite-treatment. Tyrosine phosphorylation of the 66 kDa protein was decreased by the treatment with herbimycin with a concomitant decrease in hemin- or arsenite-dependent induction of HO-1 mRNA. Tyrosine phosphorylation of a 66 kDa protein in human skin fibroblasts was also enhanced by treatment with hemin (Masuya and Taketani, unpublished observations). However, there were many major phosphotyrosyl proteins in control HeLa cells, and no changes were found on hemin- or arsenite-treatment. In line with the results of immunoblotting with anti-phosphotyrosine antibodies, immunoprecipitation with these antibodies of extracts of hemin- or arsenite-treated HeLa cells, followed by immunoblotting with the same antibody did not show any changes in phosphoproteins. Furthermore, we tried to identify the 66 kDa protein as some already known phosphotyrosyl protein with a molecular mass of 60–70 kDa, using commercially available antibodies, including anti-ZAP70, Sam68, Syk, and PTP-1 antibodies, but no antibody so far examined reacted with the enhanced 66 kDa phosphoprotein. At the present time, there is no direct evidence of a relationship between activation of the human HO-1 gene and enhanced phosphorylation of the 66 kDa protein, but our results showing that the enhancement of phosphorylation occurred prior to the induction of HO-1 mRNA, and both were inhibited by tyrosine kinase inhibitors suggest that phosphorylation of the 66 kDa protein may play a role in a signal transduction pathway that activates the human HO-1 gene, and may serve as a framework for further studies to elucidate the specific function of this protein.

We wish to thank Dr. S. Shibahara for providing the pHHO-1, Dr. J. Fujisawa for providing the anti-phosphotyrosine (4G10) and anti-Sam68 antibodies, Dr. T. Okumura for the valuable advice, and Dr. H. Kohno for reading the manuscript.

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