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The Rat Heme Oxygenase-1 Gene Is Transcriptionally Induced via the Protein Kinase A Signaling Pathway in Rat Hepatocyte Cultures

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ABSTRACT

Heme oxygenase-1 (HO-1) is the inducible form of the rate-limiting enzyme of heme degradation; it regulates the cellular content of heme. To investigate the role of the cAMP-dependent protein kinase (PKA) signaling pathway on hepatic HO-1 gene expression, primary rat hepatocyte cultures were treated with the PKA-stimulating agents dibutyryl-cAMP (Bt_2cAMP), forskolin, and glucagon. HO-1 mRNA levels were induced by these agents in a time-dependent manner with a transient maximum after 6 hr of treatment. The induction of HO-1 was dose dependent, reaching a maximum at concentrations of 250 μM Bt_2cAMP and 50 nM glucagon, respectively. The accumulation of HO-1 mRNA correlated with increased levels of HO-1 protein as determined by Western blot analysis. The Bt_2cAMP-dependent induction of HO-1 mRNA expression was prevented by pretreatment with the PKA inhibitor KT5720 but not with the protein kinase G inhibitor KT5823. HO-1 mRNA induction by $CdCl_2$ and heme was differentially affected by Bt_2cAMP. Up-regulation of the HO-1 gene by Bt_2cAMP occurred on the transcriptional level as determined by nuclear run-off assay and blocking of the Bt_2cAMP-dependent induction of HO-1 mRNA by actinomycin D. Treatment with Bt_2cAMP increased the half-life of HO-1 mRNA from 4.7 to 5.5 hr. Taken together, the results of the current study demonstrate that HO-1 gene expression is induced by activation of the cAMP signal transduction pathway via a transcriptional mechanism in primary rat hepatocyte cultures.

HO is the rate-limiting enzymatic step of heme degradation, during which it produces biliverdin subsequently converted to bilirubin by biliverdin reductase (Tenhunen *et al.*, 1968). Two genetically distinct isozymes of HO have been identified, of which HO-1 is the inducible form and HO-2 is the noninducible form (Maines, 1988). Because HO-1 is up-regulated not only by its substrate heme but also by various stress stimuli, such as UV light, heavy metals, or heat stress, HO-1 is thought to participate in general cellular defense mechanisms against oxidative stress in mammalian cells (Keyse and Tyrrell, 1989; Applegate *et al.*, 1991). This view is supported by other studies that have shown that HO-1 induction mediates an adaptive response against oxidative damage (Nath *et al.*, 1992). Moreover, HO is assumed to be a significant biological antioxidant because HO enzymatically degrades the pro-oxidant heme and generates bilirubin, a metabolite with antioxidant properties (Stocker *et al.*, 1987).

It is well recognized that the expression of the HO-1 gene is induced by signals that mediate their action via protein kinase C or prostaglandins (Muraosa and Shibahara, 1993; Koizumi *et al.*, 1995). In contrast, limited information is available on the regulation of the HO-1 gene by the PKA-signaling pathway. The elevation of the intracellular levels of the second messenger cAMP by a large number of hormones and other extracellular stimuli and the resulting activation of the PKA have been reported to either stimulate or repress genes, suggesting that complex, cell-specific molecular mechanisms may be operative in the PKA-signaling pathway (Lalli and Sassone-Corsi, 1994). Therefore, we investigated the effects of raised cAMP levels on HO-1 gene expression.

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ABBREVIATIONS: HO, heme oxygenase; Bt_2cAMP, dibutyryl cAMP; CRE, cAMP response element; BSA, bovine serum albumin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; PCK, phosphoenolpyruvate carboxykinase; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; bp, base pair(s); AP, activator protein; RE, regulatory element.

Whole liver and chicken embryo hepatocyte cultures have been used for previous studies on HO enzyme regulation (Bakken *et al.*, 1972; Sardana *et al.*, 1985); however, the role of cAMP and PKA in HO-1 gene expression has not been investigated in primary rat hepatocyte cultures. In the current study, we show that HO-1 gene expression is induced by Bt_2cAMP and other PKA-stimulating agents. The cAMP-dependent HO-1 induction was specifically regulated by activation of the PKA and occurred on the transcriptional level.

Experimental Procedures

Materials. Media 199, Dulbecco's modified Eagle's medium, and RPMI 1640 were obtained from Gibco Life Technologies (Eggenstein, Germany). Radioisotopes, the 5′-end labeling kit, and the enhanced chemiluminescence detection kit for Western blotting were from Amersham-Buchler (Braunschweig, Germany). Nitrocellulose filters were purchased from Schleicher & Schuell (Dassel, Germany). The nucleotide removal kit was from Qiagen (Studio City, CA). The multiprime labeling kit and restriction endonucleases were from New England Biolabs (Beverly, MA). Tissue culture dishes were from Falcon (Cowley, UK). All other chemicals were purchased from Sigma Chemie (Deisenhofen, Germany) and Boehringer-Mannheim Biochemica (Mannheim, Germany).

Cell culture. Hepatocytes were isolated from male Wistar rats through circulating perfusion with collagenase under sterile conditions as described previously (Muller-Eberhard *et al.*, 1988). The cells were cultured under air/CO_2 (19:1) in Medium 199 with Earle's salts containing 2 g/liter BSA, 20 mM $NaHCO_3$, 10 mM HEPES, 117 mg/liter streptomycin sulfate, 60 mg/liter penicillin, 1 nM insulin, and 10 nM dexamethasone. Fetal calf serum (5%) was present during the plating phase up to 4 hr, and cell cultures were incubated in serum-free medium for an additional 18 hr before treatment. Hepa 1–6 and NIH-3T3 cells were from American Type Culture Collection (Rockville, MD). Hepa 1–6 cells were cultured in RPMI 1640 medium containing 2% fetal calf serum, and NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum until confluency of cell monolayers was reached. Confluent monolayers were incubated in serum-free medium for 18 hr before treatment.

Determination of cellular cAMP levels. cAMP levels in cell cultures were determined with a competitive protein binding technique by using an assay kit from Amersham-Buchler.

RNA isolation, Northern blot analysis, and hybridization. Total RNA for Northern blotting was isolated as described previously (Immenschuh *et al.*, 1995). Equal quantities of RNA were separated on 1.2% agarose/2.2 M formaldehyde gels. After electrophoresis, RNA was blotted onto BAS 85 nitrocellulose membranes and baked at 80° for 4 hr. After prehybridization for 3–4 hr at 42°, blots were hybridized overnight with α-^{32}P-dCTP-radiolabeled cDNA probes at 42°. The hybridization solution contained 6× SSC, 5× Denhardt's solution (0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, and 0.2% BSA), 0.5% SDS, 50% formamide, and 100 μg/ml denatured salmon sperm DNA. Blots subsequently were washed once with 2× SSC/0.1% SDS and twice with 0.1× SSC/0.1% SDS at 65°. Filters were exposed for ≤ 4 days to X-ray films (X-OMAT RP, Kodak). Autoradiographs were quantified by densitometry using Gelimage software (Pharmacia, Vienna, Austria) or a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). When nitrocellulose filters were sequentially hybridized with different cDNA probes, the ^{32}P-labeled cDNA was removed after autoradiography through two washing steps with boiling 0.05× SSC/0.1% SDS for 15 min before rehybridization.

cDNA probes. The probes were the cDNAs of rat HO-1 and rat GAPDH as described previously (Immenschuh *et al.*, 1995) as well as the rat PCK cDNA (Kietzmann *et al.*, 1993). The cDNAs were labeled according to the oligomer method with α-^{32}P-dCTP using the multiprime DNA labeling kit according to the manufacturer's instructions.

Isolation of nuclei from rat hepatocyte cultures. Approximately 1×10^7 cells from primary rat hepatocyte cultures were washed twice with ice-cold buffer A (320 mM sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 100 μM EDTA, 100 μM phenylmethylsulfonyl fluoride, 150 μM spermine, 500 μM spermidine, 1 mM dithiothreitol, 10 mM Tris·HCl, pH 8.0). The cells were scraped off the dishes into buffer A and homogenized in a 2-ml Dounce homogenizer at 4° as described previously (Reuner *et al.*, 1995). After the addition of 4 ml of buffer A, the nuclei were pelleted by centrifugation at 300 × *g* for 5 min. The pellets were resuspended in 0.4 ml of buffer A, and the suspension was mixed with 1.6 ml of buffer B (2 M sucrose, 5 mM magnesium acetate, 100 μM EDTA, 100 μM phenylmethylsulfonyl fluoride, 150 μM spermine, 500 μM spermidine, 1 mM dithiothreitol, and 10 mM Tris·HCl, pH 8.0). This suspension was layered onto a cushion of 2 ml of buffer B and pelleted for 1 hr in a Beckman Instruments (Palo Alto, CA) SW60 rotor at 20,000 rpm and 4°. The pelleted nuclei were suspended in 25 ml of buffer C (25% glycerol, 5 mM magnesium acetate, 100 μM EDTA, 100 μM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol, and 50 mM Tris·HCl, pH 8.0).

Nuclear run-off transcription assay. The nuclear run-off reaction was performed with 2×10^6 nuclei in a volume of 20 μl as described previously (Immenschuh *et al.*, 1994) with minor modifications. The *in vitro* transcription reaction was started by the addition of 30 ml of solution D (58% glycerol, 150 mM NH_4Cl, 8.3 mM $MgCl_2$, 830 μM $MnCl_2$, 70 μM EDTA, 25 units of ribonuclease inhibitor, 830 μM ATP, 830 μM CTP, 830 μM GTP, 100 μCi of [^{32}P]UTP, 33 mM HEPES, pH 8.0). After incubation of nuclei for 30 min at 37°, the reaction was stopped by the addition of EDTA.

RNA extraction, prehybridization, and hybridization were performed as described previously (Reuner *et al.*, 1995). In brief, prehybridization was performed in hybridization solution for 12 hr at 42°, followed by hybridization for 72 hr at 42° using the rat HO-1 and GAPDH cDNAs immobilized on nitrocellulose membrane. As a control for the hybridization specificity, linearized pBR322 plasmid DNA was used. Posthybridization washes were performed in decreasing concentrations of SSC solution.

Western blot analysis. After washing of cell cultures twice with 0.9% NaCl, total protein was prepared from 1×10^6 hepatocytes by the addition of 1 ml of boiling lysis buffer (0.1% SDS, 10 mM Tris, pH 7.4) and subsequent scraping of the cells. Cells then were boiled for 5 min and homogenized by being passed through a 25-gauge needle. The homogenate was centrifuged for 5 min at 4°, and the protein content was determined in the supernatant according to the Bradford method. Forty micrograms of total protein was loaded onto a 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes by electrophoresis. Membranes were blocked with TBS buffer containing 1% BSA, 10 mM Tris·HCl, pH 7.5, and 0.1% Tween for 1 hr at room temperature. The primary antibody for HO-1 (Stress Gene, Victoria, Canada) was added in a 1:1000 dilution, and the blot was incubated for 12 hr at 4°. The enhanced chemiluminescence Western blotting system was used for detection.

Results

Time- and dose-dependent induction of HO-1 gene expression by PKA-stimulating agents in primary rat hepatocytes. To examine whether the expression of the HO-1 gene is regulated by PKA, primary cultures of rat hepatocytes were treated with the PKA activator Bt_2cAMP. At various times during Bt_2cAMP treatment, total cellular RNA was isolated. Northern blot analysis showed that Bt_2cAMP elicited a 21-fold induction of the HO-1 mRNA content, whereas it did not affect the level of GAPDH mRNA (Fig. 1). Therefore, the GAPDH gene was used throughout the study as a reference for selective induction and for normalization of the HO-1 mRNA levels. The up-regulation of HO-1 mRNA was time dependent, with a maximum level of

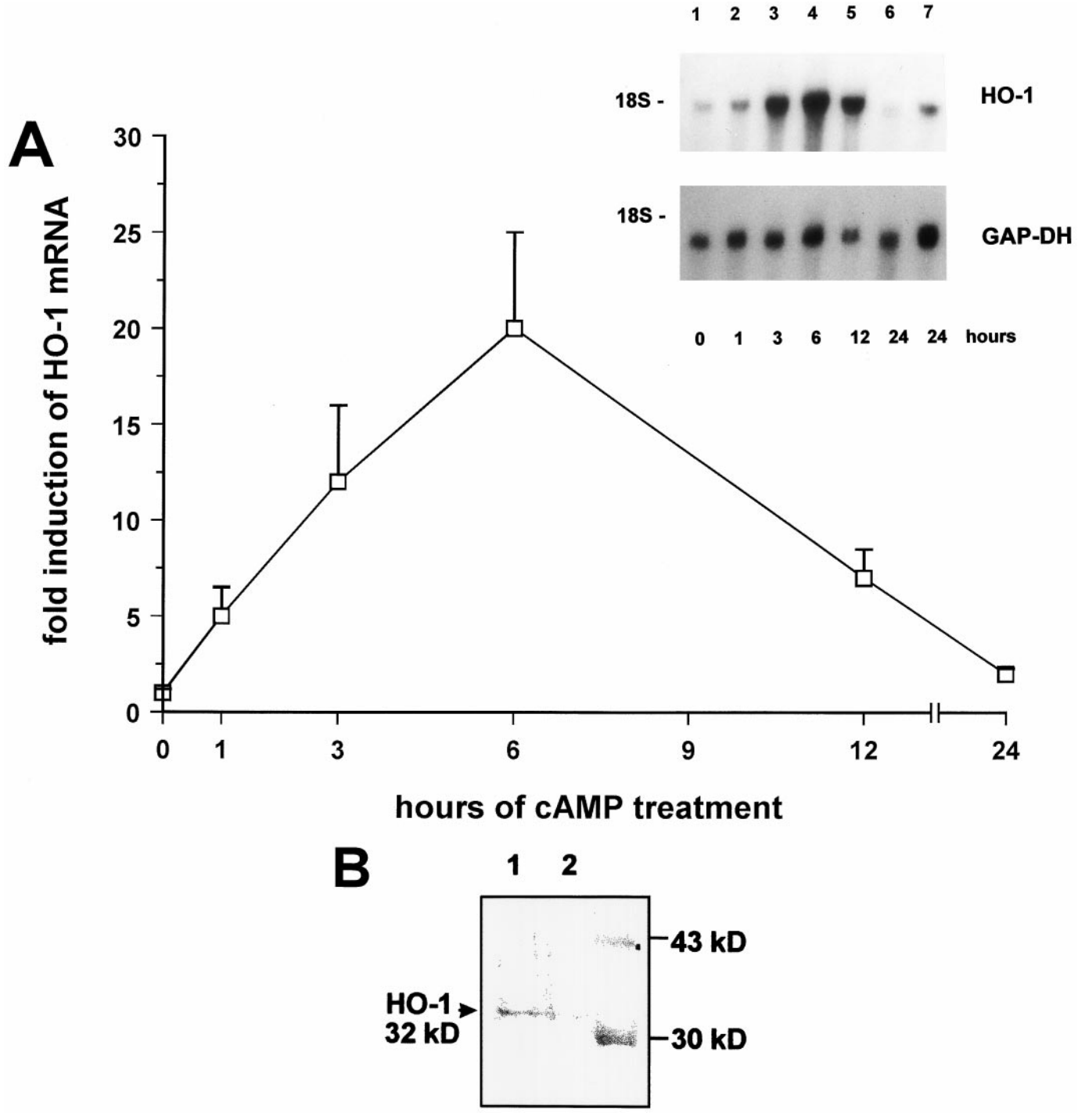

Fig. 1. Time course of HO-1 mRNA expression in primary rat hepatocytes treated with Bt_2cAMP and Western blot analysis for HO-1 protein. Primary rat hepatocytes were isolated and cultured as described in Experimental Procedures. A, Hepatocytes were treated for 18 hr with serum-free medium before cell culture was continued in the presence (*lanes 2–6*) or absence (*lane 7*) of Bt_2cAMP (250 μM) for the times indicated. Total cellular RNA (15 μg) was subjected to Northern blot analysis, probed with the ^{32}P-labeled cDNA of HO-1, and subsequently probed with the cDNA of GAPDH. The size marker was the 18S ribosomal RNA band. Autoradiograms were quantified by densitometry, and the signal of the GAPDH band served as an internal standard. Values represent the induction rate of HO-1 mRNA from at least three independent experiments and indicate the fold induction rate relative to the basal HO-1 mRNA expression at 0 hr (mean ± standard error). B, Cytosol from nontreated rat hepatocyte cultures (*lane 1*) or cultures treated with Bt_2cAMP (250 μM) for 12 hr (*lane 2*) was subjected to Western blot analysis with an antibody for rat HO-1 protein as described in Experimental Procedures. Blot is representative of three studies.

induction after 6 hr of treatment, and returned to basal expression levels after 24 hr. HO-1 expression also was induced by Bt_2cAMP on the protein level as determined by Western blot analysis (Fig. 1B). The increase in HO-1 mRNA levels during Bt_2cAMP treatment was dose dependent, reaching a peak of induction at a concentration of 250 μM Bt_2cAMP (Fig. 2).

HO-1 mRNA gene expression also was induced by glucagon, a hormone that stimulates adenylate cyclase via a receptor-mediated mechanism, which in turn produces in-

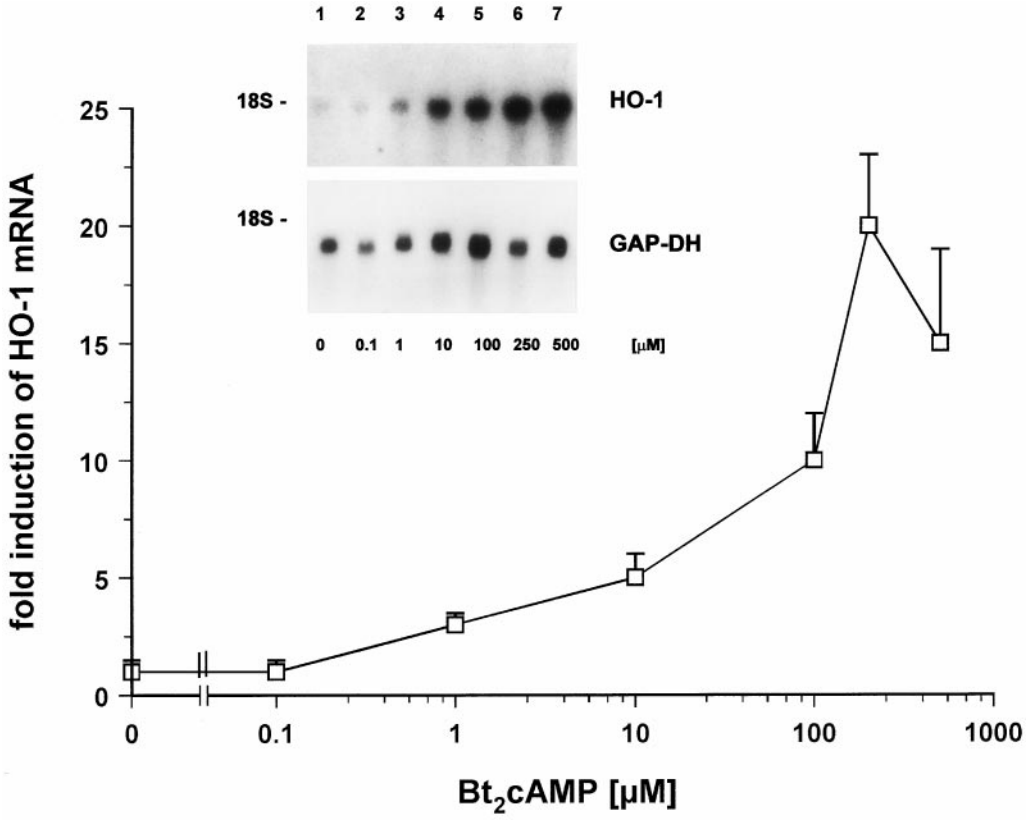

Fig. 2. Dose-dependency of HO-1 mRNA expression in primary rat hepatocytes treated with Bt_2cAMP. After isolation of primary rat hepatocytes, cell cultures were incubated for 18 hr in serum-free medium and for 6 hr in the absence (*lane 1*) or presence (*lanes 2–7*) of increasing concentrations of Bt_2cAMP (0.1–500 μM). Northern blot analysis of 15 μg of total cytosolic RNA was performed and probed with the ^{32}P-labeled HO-1 cDNA and subsequently GAPDH cDNA of. Values represent the fold induction rate of HO-1 mRNA normalized to GAPDH mRNA and are from three or four independent experiments (mean ± standard error).

creased levels of cellular cAMP (Kietzmann *et al.*, 1993). A dose-response curve for the glucagon-dependent up-regulation of HO-1 mRNA levels (Fig. 3A) shows a maximum of glucagon-dependent HO-1 mRNA expression at a concentration of 50 nM. The HO-1 mRNA time course of induction during glucagon treatment of rat hepatocytes was similar to that elicited by Bt_2cAMP, reaching a peak level at 6 hr (Fig. 3B; see also Fig. 1). For comparison, the time course of the glucagon-dependent mRNA induction of the PCK, which is the enzyme that catalyzes the rate-limiting step of gluconeogenesis, is shown in Fig. 3. The time-dependency of PCK induction is distinct from that of HO-1 in that the maximum PCK mRNA level is reached 3 hr after glucagon treatment. A time-dependent induction pattern of HO-1 mRNA with a peak level at 6 hr also was observed for the PKA-stimulating agent forskolin and the β_2-sympathomimetic terbutalin (data not shown).

HO-1 mRNA expression during treatment with Bt_2cAMP, forskolin, or glucagon also was investigated in Hepa 1–6 hepatoma and NIH-3T3 fibroblast cells. The Hepa 1–6 hepatoma cell line is a cell culture system that has been applied in previous studies; the HO-1 gene was induced by various heavy metals and heme (Alam *et al.*, 1989; Alam and Smith, 1992). Surprisingly, we observed no induction of HO-1 mRNA during treatment with Bt_2cAMP, glucagon, or forskolin in either cell line (data not shown). No difference in cellular cAMP levels was found in these two cell lines compared with that of primary rat hepatocyte cultures (data not shown). The findings indicate a cell-specific regulatory pattern for the HO-1 gene in response to the cAMP signal in primary rat hepatocyte cultures.

Inhibition of cAMP-dependent HO-1 mRNA induction by $CdCl_2$ and the PKA inhibitor KT5720. Heme and the heavy metal salt $CdCl_2$ are among the most potent inducers of the HO-1 gene so far characterized (Sardana *et al.*, 1985; Maines, 1988; Alam *et al.*, 1989; Applegate *et al.*, 1991). In Fig. 4, the HO-1 mRNA induction rate by heme and $CdCl_2$ in rat hepatocyte cultures is compared with that by Bt_2cAMP, glucagon, and forskolin. The HO-1 mRNA inducibility by heme or $CdCl_2$ exceeded that elicited by Bt_2cAMP, forskolin, or glucagon (Fig. 4).

The time course of HO-1 mRNA induction by PKA stimulation seems to be similar to that elicited by heme or $CdCl_2$ in rat hepatocyte cultures, reaching a peak mRNA level at 6 hr, as shown previously (Immenschuh *et al.*, 1995; see also Fig. 1A). Therefore, we asked whether the HO-1 gene is induced with an identical (or distinct) pattern by heme or $CdCl_2$ and Bt_2cAMP. To answer this question, cell cultures were treated with Bt_2cAMP, heme, and $CdCl_2$ alone or with combinations of these agents. Simultaneous treatment of cell cultures with heme and Bt_2cAMP elicited a prolonged induction of HO-1 mRNA levels up to 12 hr, whereas simultaneous treatment of hepatocytes with $CdCl_2$ and Bt_2cAMP showed a lower HO-1 mRNA induction level compared with that by $CdCl_2$ alone (Table 1).

To investigate the specificity of the Bt_2cAMP-dependent HO-1 gene induction inhibitors of either PKA or PKG, KT5720 and KT5823, respectively, were used in the following experiments. Hepatocyte cultures were preincubated for 30 min with KT5720 or KT5823, respectively, at a concentration of 1 μM before treatment with Bt_2cAMP. When Bt_2cAMP was added at a concentration of 10 μM, the HO-1 mRNA induction was completely prevented by KT5720. However, KT5720 did not affect the heme-dependent HO-1 mRNA induction. KT5823 showed no inhibition of the Bt_2cAMP- and heme-dependent HO-1 mRNA induction (Table 1).

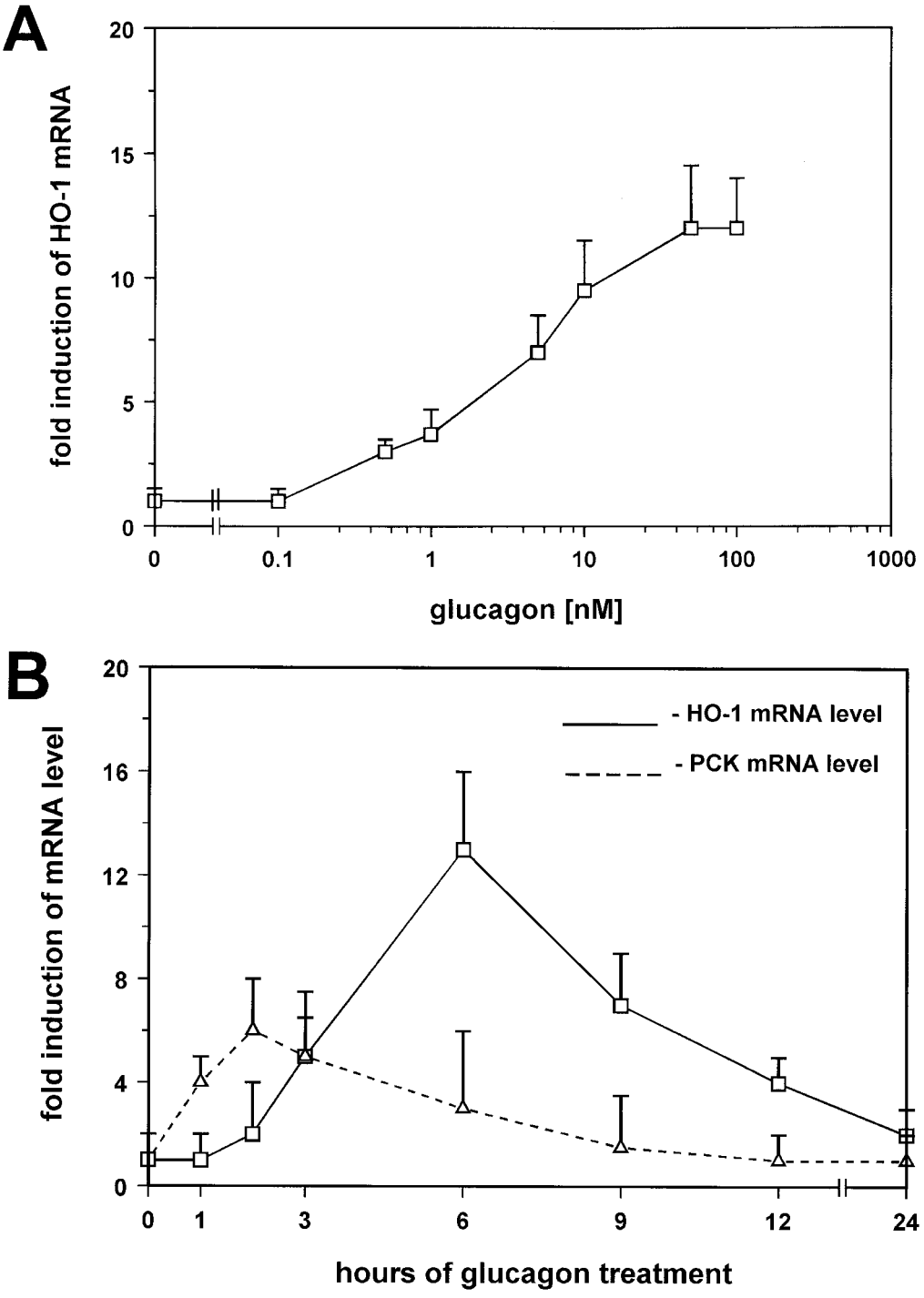

Fig. 3. Dose-dependency of HO-1 mRNA levels in primary rat hepatocytes by glucagon: time course of HO-1 and PCK mRNA expression for treatment with glucagon. Hepatocytes were cultured as described in Experimental Procedures. After 18 hr in serum-free medium, cell culture was continued (A) with increasing concentrations of glucagon (0.1–100 nM) for 6 hr or (B) in the absence and the presence of glucagon (50 nM) for the times indicated. Total cellular RNA (15 μg) was analyzed by Northern blot and probed sequentially with the ^{32}P-labeled cDNAs of HO-1, PCK, and GAPDH. Values show the rate of induction of HO-1 and PCK mRNA levels normalized to mRNA levels of GAPDH for three independent experiments (mean ± standard error).

Taken together, these results indicate that the cAMP-dependent HO-1 induction is differentially affected by the HO-1 inducers heme and $CdCl_2$. The Bt_2cAMP-dependent HO-1 mRNA induction seems to be mediated by a specific activation of the PKA, but not of the PKG, pathway.

Actinomycin D and cycloheximide inhibit the Bt_2cAMP-dependent HO-1 mRNA induction. To probe into the mechanism of the cAMP-dependent HO-1 mRNA induction, hepatocytes were treated with actinomycin D and cycloheximide before the addition of Bt_2cAMP. Exposure of cell cultures to actinomycin D effectively inhibits the rate of transcription, whereas exposure to cycloheximide suppresses the synthesis of protein (Fig. 5). Pretreatment of rat hepatocytes with actinomycin D (1 μg/ml) inhibited the Bt_2cAMP-dependent HO-1 mRNA induction. Cycloheximide (1 μg/ml) also inhibited the induction of HO-1 mRNA but to a lesser degree than that caused by actinomycin D. Subsequently, the rate of HO-1 mRNA turnover after stimulation with Bt_2cAMP was determined. As demonstrated in Fig. 6, the half-lives of HO-1 mRNA in hepatocyte cultures treated with

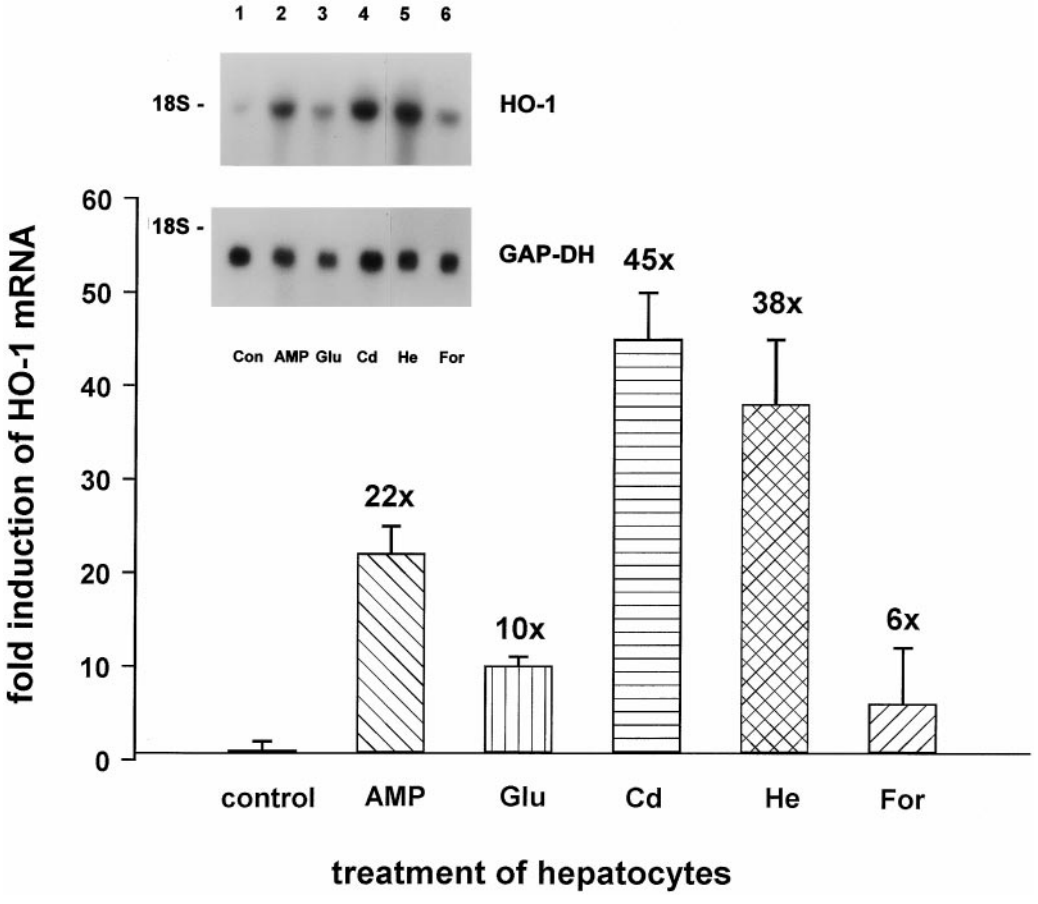

Fig. 4. Effect of Bt_2cAMP, forskolin, glucagon, heme, and $CdCl_2$ on the levels of HO-1 mRNA expression in primary rat hepatocytes. Primary rat hepatocytes were cultured as described in Experimental Procedures. After 18 hr in serum-free medium, cell culture was continued for 6 hr in the absence (*lane 1*) or the presence of 250 μM Bt_2cAMP (AMP, *lane 2*), 0.05 μM glucagon (*Glu*, *lane 3*), 10 μM $CdCl_2$ (*Cd*, *lane 4*), 10 μM heme BSA (*He*, *lane 5*), and 10 μM forskolin (*For*, *lane 6*). Total RNA (15 μg) was analyzed by Northern blotting and sequentially probed with the ^{32}P-labeled cDNAs of HO-1 and GAPDH. *Numbers above bars* represent the fold induction rate of HO-1 mRNA expression normalized to the mRNA expression levels of GAPDH and are from at least three independent experiments (mean ± standard error).

TABLE 1

Comparative effects of treatment with heme, $CdCl_2$, KT5720, and KT5823 on the Bt_2cAMP-dependent HO-1 mRNA induction in primary cultures of rat hepatocytes

Rat hepatocytes were treated for 18 hr with serum-free medium before cell culture was continued in the presence of various agents for 6 hr or 12 hr. Total RNA was isolated as described in Experimental Procedures and subjected to Northern blot analysis. Values represent the fold induction rate relative to the basal HO-1 mRNA expression at 0 hr normalized to the GAPDH mRNA levels of at least three independent experiments (mean ± standard error).

Treatment	Fold induction of HO-1 mRNA	
	6 hr	12 hr
Control	1 ± 0.2	1 ± 0.3
Bt_2cAMP (10 μM)	7 ± 0.5	2 ± 0.2
Bt_2cAMP (100 μM)	25 ± 3	7 ± 1
Heme (10 μM)	45 ± 5	15 ± 2
Bt_2cAMP (10 μM) + heme (10 μM)	42 ± 3	40 ± 4
$CdCl_2$ (10 μM)	49 ± 6	20 ± 2.5
Bt_2cAMP (10 μM) + $CdCl_2$ (10 μM)	29 ± 6	15 ± 2
KT5720 (1 μM)	1 ± 0.2	
KT5720 (1 μM) + Bt_2cAMP (10 μM)	0.7 ± 0.2	
KT5720 (1 μM) + Bt_2cAMP (100 μM)	12 ± 1	
KT5720 (1 μM) + heme (10 μM)	41 ± 4	
KT5823 (1 μM)	1 ± 0.1	
KT5823 (1 μM) + Bt_2cAMP (100 μM)	24 ± 3	
KT5823 (1 μM) + heme (10 μM)	44 ± 6	

Bt_2cAMP (≈5.5 hr) or heme (≈6.2 hr) were increased slightly compared with the HO-1 mRNA half-life under control conditions (≈4.7 hr).

HO-1 mRNA expression is induced transcriptionally by Bt_2cAMP. The prevention of the cAMP-dependent HO-1 mRNA induction by actinomycin D indicates that HO-1 gene induction occurs on the transcriptional level. Therefore, the transcription rate of the HO-1 gene in primary rat hepatocytes was determined by nuclear run-off transcription assay during treatment with Bt_2cAMP (250 μM). As shown in Fig. 7, the HO-1 gene transcription rate was increased 23 ± 3-fold [three independent experiments (mean ± standard error)] after 3 hr and remained elevated after 6 hr. The transcriptional rate of the GAPDH gene served as a control. Similar to the GAPDH mRNA regulation patterns observed during Bt_2cAMP treatment (Figs. 1 and 2), the transcription rate of GAPDH was not affected by Bt_2cAMP treatment of rat hepatocytes (Fig. 7).

Discussion

The major findings of this study of HO-1, the inducible form of the rate-limiting enzyme of heme degradation (Tenhunen *et al.*, 1968), are that (1) mRNA expression of the HO-1 gene is induced by Bt_2cAMP and other PKA-stimulating agents in primary rat hepatocytes cultures, (2) up-regulation of HO-1 mRNA expression by cAMP is prevented by the PKA inhibitor KT5720 but not the PKG inhibitor KT5823, and (3) the cAMP-dependent HO-1 induction occurs on the transcriptional level.

It has been reported that hepatic HO enzyme activity is induced *in vivo* during treatment of rats with various hormones such as glucagon, insulin, and epinephrine (Bakken *et al.*, 1972). Others have shown that Bt_2cAMP and glucagon inhibit the basal and $CoCl_2$-induced HO enzyme activity in cultured chicken embryo hepatocytes (Sardana *et al.*, 1985). The latter finding is not in agreement with the data for our

study in primary rat hepatocyte cultures that show a significant Bt_2cAMP- and glucagon-dependent HO-1 mRNA induction (Figs. 1–4). These conflicting data on HO regulation by cAMP in adult rat versus chicken embryo hepatocyte cultures may occur for two reasons. First, they may represent a species-specific difference between rat and chicken regarding the hepatic responsiveness to the cAMP signal. Second, the response to the PKA-signaling pathway may be affected by developmental changes in liver function for embryonic and adult hepatocytes (Sardana *et al.*, 1985). The induction of the HO-1 gene by PKA stimulation in primary rat hepatocytes seems to be a cell-specific response. In neither Hepa 1–6 cells, a mouse hepatoma cell line that has been used in studies on HO-1 gene regulation (Alam *et al.*, 1989; Alam and Smith, 1992) nor NIH-3T3 fibroblast cells has HO-1 gene expression been affected by PKA-stimulating agents (data not shown). However, significant cAMP-dependent induction of HO-1 mRNA expression was observed in our primary rat hepatocyte cell culture system (Figs. 1–4). Because no difference in cellular levels of cAMP was observed in Hepa 1–6 cells, NIH-3T3 cells, or rat hepatocyte cultures, it is conceivable that the PKA-signaling pathway (e.g., PKA activity) may not be equally functional in the various cell culture models. Interestingly, Durante *et al.* (1997) recently demonstrated that the HO-1 gene is induced by cAMP in vascular smooth muscle cell cultures.

The induction of the HO-1 gene by various stimuli has been demonstrated previously (Shibahara *et al.*, 1987; Alam *et al.*, 1989; Keyse and Tyrrell, 1989; Applegate *et al.*, 1991; Nath *et al.*, 1992; Koizumi *et al.*, 1995). Therefore, we explored in the current study whether the PKA-signaling pathway in primary rat hepatocytes interferes with that of other HO-1 inducers. Two of the most potent inducers of the HO-1 gene expression are the HO substrate heme and the heavy metal salt $CdCl_2$ (Maines, 1988; Sardana *et al.*, 1985; Alam *et al.*, 1989; Applegate *et al.*, 1991). As shown in Fig. 4 and Table 1, the maximum rate of HO-1 mRNA induction by heme and $CdCl_2$ is higher than that elicited by Bt_2cAMP, forskolin, or glucagon. Data on the treatment of hepatocytes with a combination of compounds (Table 1) indicate that Bt_2cAMP has differential effects on HO-1 mRNA induction by heme and $CdCl_2$. Interestingly, treatment with Bt_2cAMP reduces the $CdCl_2$-dependent induction of HO-1 mRNA. Although one could speculate that these two agents mediate their effects on HO-1 expression via similar signaling pathways, the data are too preliminary for such a conclusion to be made. The mechanism or mechanisms of HO-1 gene induction by heme or heavy metals are still unknown; however, two hypotheses, based on *in vivo* and *in vitro* observations, have been proposed. First, the administration of heme or heavy metals may increase the intracellular levels of reactive oxygen intermediates (Llesuy and Tomaro, 1994), which in turn may function as second messengers for the activation of a variety of genes (Schreck *et al.*, 1991). Second, HO-1 induction by $CdCl_2$ may be mediated by a modification of the cellular glutathione level, which is decreased by various heavy metals (Applegate *et al.*, 1991).

Stimulation of the HO-1 gene by most, if not all, inducers is controlled primarily at the transcriptional level (Shibahara *et al.*, 1987; Alam *et al.*, 1989; Takeda *et al.*, 1994), which is governed by *cis*-acting elements of the HO-1 promoter 5′-flanking region (for a review, see Choi and Alam, 1996). So far, several REs of three species (human, mouse, and rat) have been characterized, such as that for the regulation by $CdCl_2$ (Takeda *et al.*, 1994), prostaglandin J_2 (Koizumi *et al.*, 1995), phorbol myrisate acetate (Muraosa and Shibahara, 1993), heme (Lavrovsky *et al.*, 1994), or hypoxia (Lee *et al.*, 1997). The cAMP-dependent HO-1 induction in rat hepatocyte cultures is mainly mediated on the transcriptional level as indicated by blocking of HO-1 mRNA induction by actinomycin D (Fig. 5), determination of HO-1 mRNA half-lives (Fig. 6), and nuclear run-off transcription assay (Fig. 7). Different classes of REs that mediate the cAMP-dependent transcriptional activation of mammalian genes are known. One class is the CRE, initially described in the somatostatin gene (Montminy *et al.*, 1986), which is the nuclear binding site of the transcription factor CRE-binding protein (Montminy and Bilezikjian, 1987). A CRE-like element was identified by computer search between −664 and −657 relative to the transcription initiation site in the rat HO-1 gene 5′-flanking region (Müller *et al.*, 1987), which matches the somatostatin CRE in 7 of 8 bp. Reporter constructs containing the −714 bp of the rat HO-1 promoter 5′-flanking region with the HO-1 CRE-like element, however, mediated only a minor response to cAMP-treatment when transiently transfected into rat hepatocyte cultures (S. Immenschuh and T. Kietzmann; unpublished observations), indicating that this CRE-like sequence of the HO-1 promoter is not the major target sequence of the PKA-signaling pathway. Another class of REs responsive to cAMP is represented by the AP-2 binding site, as demonstrated for the metallothionein 2A gene (Imagawa *et al.*, 1987) and the acetyl carboxylase gene (Park and Kim, 1993). In addition, the CGTCA sequence motif has been demonstrated to be involved in the cAMP-dependent transcriptional regulation of the vasoactive intestinal peptide gene (Fink *et al.*, 1988). No consensus sequences matching the AP-2 binding site or the CGTCA motif were identified within the first 1300 bp of the HO-1 promoter 5′-flanking

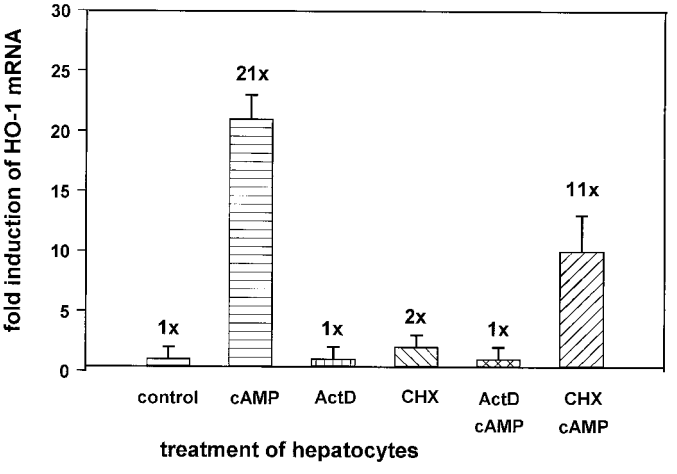

Fig. 5. Effect of treatment with actinomycin D and cycloheximide on Bt_2cAMP-dependent induction of HO-1 mRNA in primary rat hepatocytes. Primary rat hepatocytes were cultured as described in Experimental Procedures. Hepatocytes were pretreated for 30 min with actinomycin D (*ActD*; 1 μg/ml) or cycloheximide (*CHX*; 1 μg/ml) as indicated. Bt_2cAMP was added, and cell culture was continued for 6 hr, after which total RNA was isolated and subjected to Northern blot analysis. Blots were probed sequentially with the ^{32}P-labeled cDNAs of HO-1 and GAPDH. *Numbers above bars* represent the fold induction rate of HO-1 mRNA normalized to GAPDH levels and are from two or three independent experiments (mean ± standard error).

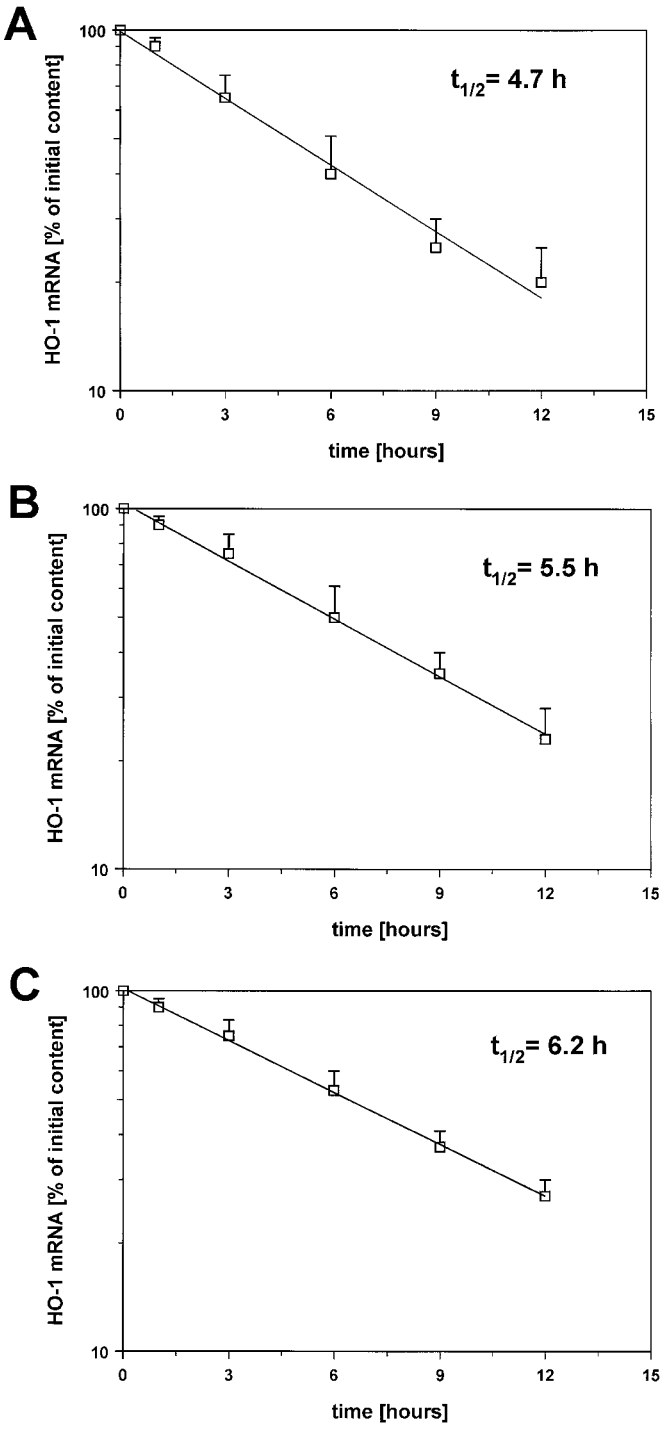

Fig. 6. Effect of Bt_2cAMP and heme on the rate of degradation of HO-1 mRNA in primary rat hepatocyte cultures. Primary rat hepatocytes were cultured as described in Experimental Procedures. Hepatocytes were cultured (A) in the absence or the presence of (B) Bt_2cAMP (250 μM) or (C) heme (10 μM) for 6 hr. Cell culture was continued with actinomycin D (1 μg/ml). Total RNA was isolated at the times indicated, and the levels of HO-1 mRNA were determined by Northern blot analysis. The main plot is a semilog plot of individual points from two independent experiments (mean ± standard error). $t_{½}$, half-lives calculated from the graphs.

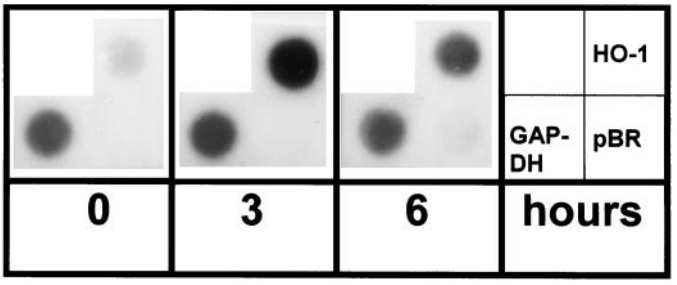

Fig. 7. Effect of Bt_2cAMP on HO-1 gene transcription in primary rat hepatocyte cultures. Primary rat hepatocytes were treated for 18 hr with serum-free medium before cell culture was continued in the presence of Bt_2cAMP (250 μM). At 0, 3, or 6 hr, nuclei were prepared and subjected to nuclear run-off transcription assays as described in Experimental Procedures. Radiolabeled nascent RNA transcripts were purified and hybridized to HO-1 and GAPDH cDNAs or pBR322 (*pBR*) immobilized on nitrocellulose paper as indicated. The plasmid pBR322 was used as a control for nonspecific hybridization. Results are from a representative experiment.

region (Müller *et al.*, 1987). It is conceivable that the maximal effect of cAMP on the transcriptional activation of the HO-1 gene is mediated by a synergism of more than one *cis*-acting element and transcription factor, as has been shown for the rat PCK gene (Roesler *et al.*, 1995). The kinetics of the HO-1 mRNA accumulation by cAMP (Fig. 1A) also could suggest that HO-1 gene induction is mediated via an indirect mechanism. One possibility may be that PKA activation induces the c-*fos* gene encoding the Fos protein, which is part of the transcription factor AP-1 (Janknecht *et al.*, 1995). AP-1 binding sites have been demonstrated previously to be involved in the transcriptional activation of the human and mouse HO-1 genes (Alam and Zhining, 1992; Muraosa and Shibahara, 1993).

The cAMP-dependent induction of the HO-1 gene is of physiological and pharmacological significance for several reasons. As judged on the basis of the gene expression pattern, HO-1 could play a biological role common to that of the metallothioneins. Metallothioneins are a family of highly conserved low-molecular-weight proteins, the main function of which seems to be the detoxification of heavy metals and attenuation of oxidant stress (Kagi, 1991). The metallothionein-1 and HO-1 genes are induced in parallel by stress stimuli such as heme, metalloporphyrins, or heavy metals (Alam and Smith; 1992). In agreement with the data of this study regarding HO-1 mRNA regulation by Bt_2cAMP, others have demonstrated a cell-specific induction by cAMP in primary rat hepatocyte cultures for the metallothionein-1 gene (Nebes *et al.*, 1988). The increase of HO-1 activity and mRNA expression seems to be a protective response against oxidative stress in various *in vivo* (Nath *et al.*, 1992) and *in vitro* (Keyse and Tyrrell, 1989; Applegate *et al.*, 1991) models. HO-1 enzymatically breaks down heme, thereby mitigating the hazardous cellular effects of the pro-oxidant heme. In addition, the HO-1 product biliverdin is converted by the enzyme biliverdin reductase to bilirubin, which is an antioxidant implicated in cellular defense functions (Stocker *et al.*, 1987). The cytoprotective effect of HO-1 has been demonstrated directly in coronary endothelial cell cultures. In this cell culture model, the toxicity caused by heme and hemoglobin was attenuated efficiently when the HO-1 cDNA was transfected stably into the cells and the HO-1 gene was overexpressed (Abraham *et al.*, 1995). Therefore, the induction of the HO-1 gene may be significant for the general endogenous cellular protection during inflammation, as has been suggested by Willis *et al.* (1996). In addition, the metabolism of heme and therefore the heme-degrading enzy-

matic activity of HO seems to be closely correlated to drug and steroid metabolism. It has been suggested that HO plays a major role in the regulation of biotransformation reactions that depend on the cytochrome P450 system, which contains heme as an essential compound (Maines, 1988). In a recent study, it was demonstrated that the phenobarbital-dependent mRNA induction of various P450 cytochrome forms (CYP2B1, CYP2B2, and CYP3A1) is repressed in primary rat hepatocytes by treatment with Bt_2cAMP, forskolin, and glucagon (Sidhu and Omiecinski, 1995). This finding regarding the regulation of P450 isozymes could correspond with the observation made in our study: the cAMP-dependent induction of HO-1 decreases the available heme pool and may affect reciprocally the synthesis of P450 isozymes.

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References

Abraham NG, Lavrovsky Y, Schwartzman ML, Stoltz RA, Levere RD, Gerritsen ME, Shibahara S, and Kappas A (1995) Transfection of the human heme oxygenase gene into rabbit coronary microvessel endothelial cells: protective effect against heme and hemoglobin toxicity. *Proc Natl Acad Sci USA* **92:**6798–6802.

Alam J, Shibahara S, and Smith A (1989) Transcriptional activation of the heme oxygenase gene by heme and cadmium in mouse hepatoma cells. *J Biol Chem* **264:**6371–6375.

Alam J and Smith A (1992) Heme-hemopexin-mediated induction of metallothionein gene expression. *J Biol Chem* **267:**16379–16384.

Alam J and Zhining D (1992) Distal AP-1 binding sites mediate basal level enhancement and TPA induction of the mouse heme oxygenase-1 gene. *J Biol Chem* **267:**21894–21900.

Applegate LA, Luscher P, and Tyrrell RM (1991) Induction of heme oxygenase: a general response to oxidant stress in cultured mammalian cells. *Cancer Res* **51:**974–978.

Bakken AF, Thaler MM, and Schmid R (1972) Metabolic regulation of heme catabolism and bilirubin production: hormonal control of hepatic heme oxygenase activity. *J Clin Invest* **51:**530–536.

Choi AMK and Alam J (1996) Heme oxygenase-1: function, regulation, and implication of a novel stress-inducible protein in oxidant-induced lung injury. *Am J Respir Cell Mol Biol* **15:**9–19.

Durante W, Christodoulides N, Cheng K, Peyton KJ, Sunahara RK, and Schafer AI (1997) cAMP induces heme oxygenase-1 gene expression and carbon monoxide production in vascular smooth muscle. *Am J Physiol* **273:**H317–H323.

Fink JS, Verhave M, Kasper S, Tsuda T, Mandel G, and Goodman RH (1988) The CGTCA sequence motif is essential for biological activity of the vasoactive intestinal peptide gene cAMP-regulated enhancer. *Proc Natl Acad Sci USA* **85:**6662–6666.

Imagawa M, Chiu R, and Karin M (1987) Transcription factor AP-2 mediates induction by two different signal transduction pathways: protein kinase C and cAMP. *Cell* **51:**251–260.

Immenschuh S, Iwahara S-I, Satoh H, Nell C, Katz N, and Muller-Eberhard U (1995) Expression of the mRNA of heme-binding protein 23 is coordinated with that of heme oxygenase-1 by heme and heavy metals in primary rat hepatocytes and hepatoma cells. *Biochemistry* **34:**13407–13411.

Immenschuh S, Nagae Y, Satoh H, Baumann H, and Muller-Eberhard U (1994) The rat and human hemopexin genes contain an identical interleukin-6 response element that is not a target of CAAT enhancer-binding protein isoforms. *J Biol Chem* **269:**12654–12661.

Janknecht R, Cahill MA, and Nordheim A (1995) Signal integration at the *c-fos* promoter *Carcinogenesis* **16:**443–450 (1995).

Kagi JHR (1991) Overview of metallothionein. *Methods Enzymol* **205:**613–626.

Keyse SM and Tyrrell RM (1989) Heme oxygenase is the major 32-kDa stress protein induced in human skin fibroblasts by UVA radiation, hydrogen peroxide, and sodium arsenite. *Proc Natl Acad Sci USA* **86:**99–103.

Kietzmann T, Schmidt H, Unthan-Fechner K, Probst I, and Jungermann K (1993) A ferro-heme protein senses oxygen levels, which modulate the glucagon-dependent activation of the phosphoenolpyruvate carboxykinase gene in rat hepatocyte cultures. *Biochem Biophys Res Commun* **195:**792–798.

Koizumi T, Odani N, Okuyama T, Ichikawa A, and Negishi M (1995) Identification of a *cis*-regulatory element for 12-prostaglandin J2-induced expression of the rat heme oxygenase gene. *J Biol Chem* **270:**21779–21784.

Lalli E and Sassone-Corsi P (1994) Signal transduction and gene regulation: the nuclear response to cAMP. *J Biol Chem* **269:**17359–17362.

Lavrovsky Y, Schwartzman ML, Levere RD, Kappas A,, Abraham NG (1994) Identification of binding sites for transcription factors NF-κB and AP-2 in the promoter region of the human heme oxygenase 1 gene. *Proc Natl Acad Sci USA* **91:**5987–5991.

Lee PJ, Jiang B-H, Chin BY, Iyer NV, Alam J, Semenza GL, and Choi AMK (1997) Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. *J Biol Chem* **272:**5375–5381.

Llesuy SF and Tomaro ML (1994) Heme oxygenase and oxidative stress: evidence for involvement of bilirubin as physiological protector against oxidative stress. *Biochim Biophys Acta* **1223:**9–14.

Maines M (1988) Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J* **2:**2557–2568.

Montminy MR, Sevarino KA, Wagner JA, Mandel G, and Goodman RH (1986) Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc Natl Acad Sci USA* **83:**6682–6686.

Montminy MR and Bilezikjian LM (1987) Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. *Nature (Lond)* **328:**175–178.

Müller RM, Taguchi H, and Shibahara S (1987) Nucleotide sequence and organization of the rat heme oxygenase gene. *J Biol Chem* **262:**6795–6802.

Muller-Eberhard U, Liem HH, Grasso JA, Giffhorn-Katz S, DeFalco MG, and Katz NR (1988) Increase in surface expression of transferrin receptors on cultured hepatocytes of adult rats in response to iron deficiency. *J Biol Chem* **263:**14753–14756.

Muraosa Y and Shibahara S (1993) Identification of a *cis*-regulatory element and putative *trans*-acting factors responsible for TPA-mediated induction of heme oxygenase expression in myelomonocytic cells. *Mol Cell Biol* **13:**7881–7891.

Nath KA, Balla G, Vercellotti GM, Balla J, Jacob HS, Levitt MD, and Rosenberg ME (1992) Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat. *J Clin Invest* **90:**267–270.

Nebes VL, DeFranco D, and Morris SM Jr (1988) Cyclic AMP induces metallothionein gene expression in rat hepatocytes but not in rat kidney. *Biochem J* **255:**741–743.

Park K and Kim KH (1993) The site of cAMP action in the insulin induction of gene expression of acetyl-CoA carboxylase is AP-2. *J Biol Chem* **268:**17811–17819.

Reuner KH, Wiederhold M, Dunker P, Just I, Bohle RM, Kröger M, and Katz N (1995) Autoregulation of actin synthesis in hepatocytes by transcriptional and posttranscriptional mechanisms. *Eur J Biochem* **230:**32–37.

Roesler WJ, Graham JG, Kolen R, Klemm DJ, and McFie PJ (1995) The cAMP response element binding protein synergizes with other transcription factors to mediate cAMP responsiveness. *J Biol Chem* **270:**8225–8232.

Sardana MK, Sassa S, and Kappas A (1985) Hormonal regulation of heme oxygenase induction in avian hepatocyte culture. *Biochem Pharmacol* **34:**2937–2944.

Schreck R, Rieber P and Baeuerle PA (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO J* **10:**2247–2258.

Shibahara S, Müller R, and Taguchi H (1987) Transcriptional control of rat heme oxygenase by heat shock. *J Biol Chem* **262:**12889–12892.

Sidhu JS and Omiecinski CJ (1995) cAMP-associated inhibition of phenobarbital-inducible cytochrome P450 gene expression in primary rat hepatocyte cultures. *J Biol Chem* **270:**12762–12773.

Stocker R, Yamamoto Y, Mc Donagh AN, Glazer AN, and Ames BN (1987) Bilirubin is an antioxidant of possible physiological significance. *Science (Washington DC)* **235:**1043–1046.

Takeda K, Ishizawa S, Sato M, Yoshida T, and Shibahara S (1994) Identification of a *cis*-acting element that is responsible for cadmium-mediated induction of the human heme oxygenase gene. *J Biol Chem* **269:**22858–22867.

Tenhunen R, Marver HS, and Schmid R (1968) The enzymatic conversion of heme to bilirubin by microsomal heme oxygenase. *Proc Natl Acad Sci USA* **61:**748–755.

Willis D, Moore AR, Frederick R, and Willoughby DA (1996) Heme oxygenase: a novel target for the modulation of the inflammatory response. *Nat Med* **2:**87–90.

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