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 (Bt2cAMP), 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 Bt2cAMP 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 Bt2cAMP-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 CdCl2 and heme was differentially affected by Bt2cAMP. Up-regulation of the HO-1 gene by Bt2cAMP occurred on the transcriptional level as determined by nuclear run-off assay and blocking of the Bt2cAMP-dependent induction of HO-1 mRNA by actinomycin D. Treatment with Bt2cAMP 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.
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_cAMP 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/CO2 (19:1) in Medium 199 with Earle's salts containing 2 g/liter BSA, 20 mM NaHCO3, 10 mM HEPES, 117 mg/liter streptomycin sulfate, 60 mg/liter penicillin, 1 μM insulin, and 10 mM 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 confluence 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 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% 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-32P-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 32P-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-32P-dCTP using the multiprime DNA labeling kit according to the manufacturer's instructions.

Isolation of nuclei from rat hepatocyte cultures. Approximately 1 × 106 cells from primary rat hepatocyte cultures were washed twice with ice-cold buffer A (320 mM sucrose, 3 mM CaCl2, 2 mM magnesium acetate, 100 μM EDTA, 100 μM phenylmethylsulfonyl fluoride, 150 μM spermine, 500 μM spermidine, 1 mM dithiotreitol, 10 mM Tris-HCl, pH 8.0). The nuclei were centrifuged off the dishes and pelleted in a 2-ml Dounce homogenizer at 4° as described previously (Reuner et al., 1995). After the addition of 4 ml of buffer B (5 mM sucrose, 5 mM magnesium acetate, 100 μM EDTA, 100 μM phenylmethylsulfonyl fluoride, 150 μM spermine, 500 μM spermidine, 1 mM dithiotreitol, 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 dithiotreitol, and 50 mM Tris-HCl, pH 8.0).

Nuclear run-off transcription assay. The nuclear run-off reaction was performed with 2 × 106 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 NH4Cl, 8.3 mM MgCl2, 830 μM MnCl2, 70 μM EDTA, 25 units of ribonuclease inhibitor, 830 μM ATP, 830 μM CTP, 830 μM GTP, 100 μM of [32P]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 × 106 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_cAMP. At various times during Bt_cAMP treatment, total cellular RNA was isolated. Northern blot analysis showed that Bt_cAMP 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
induction after 6 hr of treatment, and returned to basal expression levels after 24 hr. HO-1 expression also was induced by Bt2cAMP on the protein level as determined by Western blot analysis (Fig. 1B). The increase in HO-1 mRNA levels during Bt2cAMP treatment was dose dependent, reaching a peak of induction at a concentration of 250 μM Bt2cAMP (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-
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 Bt2cAMP, 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 β2-sympathomimetic terbutalin (data not shown).

HO-1 mRNA expression during treatment with Bt2cAMP, 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 Bt2cAMP, glucagon, or forskolin in the Hepa 1–6 hepatoma 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 CdCl2 and the PKA inhibitor KT5720. Heme and the heavy metal salt CdCl2 are among the most potent inducers of the HO-1 gene so far characterized (Sardana et al., 1985; Maine, 1988; Alam et al., 1989; Applegate et al., 1991). In Fig. 4, the HO-1 mRNA induction rate by heme and CdCl2 in rat hepatocyte cultures is compared with that by Bt2cAMP, glucagon, and forskolin. The HO-1 mRNA inducibility by heme or CdCl2 exceeded that elicited by Bt2cAMP, 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 CdCl2 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 CdCl2 and Bt2cAMP. To answer this question, cell cultures were treated with Bt2cAMP, heme, and CdCl2 alone or with combinations of these agents. Simultaneous treatment of cell cultures with heme and Bt2cAMP elicited a prolonged induction of HO-1 mRNA levels up to 12 hr, whereas simultaneous treatment of hepatocytes with CdCl2 and Bt2cAMP showed a lower HO-1 mRNA induction level compared with that by CdCl2 alone (Table 1).

To investigate the specificity of the Bt2cAMP-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 Bt2cAMP. When Bt2cAMP 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 Bt2cAMP- and heme-dependent HO-1 mRNA induction (Table 1).
Taken together, these results indicate that the cAMP-dependent HO-1 induction is differentially affected by the HO-1 inducers heme and CdCl₂. The Bt₂cAMP-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 Bt₂cAMP-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 Bt₂cAMP. 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 Bt₂cAMP-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 Bt₂cAMP was determined. As demonstrated in Fig. 6, the half-lives of HO-1 mRNA in hepatocyte cultures treated with...
This page contains a discussion on the regulation of HO-1 gene expression in primary rat hepatocytes. The focus is on the role of cAMP in inducing HO-1 mRNA expression and the effects of various agents on this process. The text includes experimental methods, results, and conclusions regarding the transcriptional regulation of the HO-1 gene.

**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 Bt2cAMP 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 Bt2cAMP and glucagon inhibit the basal and CoCl2-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

**Table 1**

Comparative effects of treatment with heme, CdCl2, KT5720, and KT5823 on the Bt2cAMP-dependent HO-1 mRNA induction in primary cultures of rat hepatocytes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fold induction of HO-1 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>Bt2cAMP (10 μM)</td>
<td>7 ± 0.5</td>
</tr>
<tr>
<td>Bt2cAMP (100 μM)</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Heme (10 μM)</td>
<td>45 ± 5</td>
</tr>
<tr>
<td>Bt2cAMP (10 μM) + heme (10 μM)</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>CdCl2 (10 μM)</td>
<td>49 ± 6</td>
</tr>
<tr>
<td>Bt2cAMP (10 μM) + CdCl2 (10 μM)</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>KT5720 (1 μM)</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>KT5720 (1 μM) + Bt2cAMP (10 μM)</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>KT5720 (1 μM) + heme (10 μM)</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>KT5720 (1 μM) + Bt2cAMP (100 μM)</td>
<td>41 ± 4</td>
</tr>
<tr>
<td>KT5823 (1 μM)</td>
<td>1 ± 0.1</td>
</tr>
<tr>
<td>KT5823 (1 μM) + Bt2cAMP (100 μM)</td>
<td>24 ± 3</td>
</tr>
<tr>
<td>KT5823 (1 μM) + heme (10 μM)</td>
<td>44 ± 6</td>
</tr>
</tbody>
</table>

Bt2cAMP (~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 Bt2cAMP.** 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 Bt2cAMP (250 μM). As shown in Fig. 7, the HO-1 gene transcription rate was increased ~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 Bt2cAMP treatment (Figs. 1 and 2), the transcription rate of GAPDH was not affected by Bt2cAMP treatment of rat hepatocytes (Fig. 7).

**Fig. 4.** Effect of Bt2cAMP, forskolin, glucagon, heme, and CdCl2 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 Bt2cAMP (AMP, lane 2), 0.05 μM glucagon (Glu, lane 3), 10 μM CdCl2 (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 32P-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).
study in primary rat hepatocyte cultures that show a significant Bt2cAMP- 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 CdCl₂ (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 CdCl₂ is higher than that elicited by Bt2cAMP, forskolin, or glucagon. Data on the treatment of hepatocytes with a combination of compounds (Table 1) indicate that Bt2cAMP has differential effects on HO-1 mRNA induction by heme and CdCl₂. Interestingly, treatment with Bt2cAMP reduces the CdCl₂-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 mechanisms 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 CdCl₂ 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 CdCl₂ (Takeda et al., 1994), prostaglandin J₂ (Koizumi et al., 1995), phorbol myristate 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 (Muller 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 (Iamage 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 region in human, mouse, or rat.
region (Muller 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 Bt2cAMP, 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-

![Fig. 6](image1.png)

**Fig. 6.** Effect of Bt2cAMP 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) Bt2cAMP (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). t½, half-lives calculated from the graphs.

![Fig. 7](image2.png)

**Fig. 7.** Effect of Bt2cAMP 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 Bt2cAMP (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.
mactive 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 Bt2cAMP, forskolin, and glucagon (Sidhu and Omiecinski, 1995). This finding regarding the regulation of P450 isoforms 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 isoforms.

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References


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