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Transcriptional Induction of Heme Oxygenase-1 Gene Expression by Okadaic Acid in Primary Rat Hepatocyte Cultures

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ABSTRACT

Heme oxygenase (HO) catalyzes the rate-limiting enzymatic step of heme degradation and regulates the cellular heme content. The gene expression of the inducible isoform of HO, HO-1, is up-regulated in response to various agents causing oxidative stress. To investigate the regulatory role of protein phosphatases in the hepatic regulation of HO-1 gene expression, primary cultures of rat hepatocytes were treated with okadaic acid (OA), which specifically inhibits the serine threonine protein phosphatases 1 and 2A. Both protein synthesis and mRNA expression of HO-1 were induced by OA in cultured hepatocytes, but not in cultured tissue macrophages of rat liver. The HO-1 mRNA induction by OA occurred in a time- and concentration-dependent manner. Simultaneous treatment with OA plus dibutyryl cAMP caused a synergistic up-regulation of steady-state levels of HO-1 mRNA, and the specific protein kinase A inhibitor KT5720 markedly reduced the OA-dependent HO-1 mRNA induction. In contrast, the dibutyryl cAMP-dependent induction of the phosphoenolpyruvate carboxykinase mRNA expression and enzyme activity was inhibited by simultaneous treatment with OA in hepatocytes. The induction of the HO-1 gene expression by OA was transcriptional as determined by studies with actinomycin D, nuclear run-off assay, and measurement of the half-life of HO-1 mRNA. Luciferase reporter constructs containing DNA sequences of the rat HO-1 promoter 5′-flanking region were up-regulated by OA in transiently transfected hepatocytes. Mutation of the cAMP response element/activator protein-1 (−665/−654) site obliterated the OA-dependent induction, suggesting that this element is involved in the transcriptional induction of the rat HO-1 gene by OA.

Heme oxygenase (HO) catalyzes the first and rate-limiting step of heme degradation and controls the cellular heme availability (Tenhunen et al., 1968). HO enzymatically breaks down the pro-oxidant heme, producing equimolar amounts of carbon monoxide, iron, and biliverdin, which are converted by biliverdin reductase into the antioxidant bilirubin (Stocker et al., 1987). At least two distinct isoforms of HO are known that are the products of different genes. In contrast to the constitutive isozyme HO-2 (Maines et al., 1986), HO-1 is the inducible isozyme, which is highly up-regulated by various stress stimuli including its substrate heme, heavy metals, UV light, lipopolysaccharide, heat shock, and hyperoxia (Shibahara et al., 1987; Applegate et al., 1991; for reviews see Maines, 1988, and Choi and Alam, 1996). Although the exact functional role of HO-1 induction is not fully understood, various researchers have shown that HO-1 provides protection against oxidative stress in various cell culture and in in vivo models (Abraham et al., 1995; Lee et al., 1996). Overexpression of the HO-1 gene attenuates the toxic effects of heme proteins in coronary endothelial cells (Abraham et al., 1995) and protects pulmonary epithelial cells against hyperoxia (Lee et al., 1996). Poss and Tonegawa (1997a) have shown that HO-1-deficient mice develop an anemia with abnormally low serum iron levels, along with an overload of iron in liver and kidney, causing oxidative damage and chronic inflammation. In addition, HO-1-deficient mice were highly susceptible to endotoxin-mediated hepatic damage, resulting in a higher mortality rate from endotoxic shock in these animals (Poss and Tonegawa, 1997b).

OA is a polyether fatty acid isolated from marine sponges

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ABBREVIATIONS: HO, heme oxygenase; ActD, actinomycin D; AP-1, activator protein-1; Bt_2cAMP, dibutyryl cAMP; CAT, chloramphenicol acetytransferase; CHX, cycloheximide; CRE, cAMP response element; CREB, CRE-binding protein; CYP, cytochrome P450; ERK, extracellular signal-regulated kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; iNOS, inducible nitric-oxide synthase; OA, okadaic acid; PCK, phosphoenolpyruvate carboxykinase; PK, protein kinase; PCR, polymerase chain reaction; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; PP, protein phosphatase; rRNA, ribosomal RNA; TF, transcription factor; PMSF, phenylmethylsulfonyl fluoride.

that initially has been shown to be a tumor promoter (Holmes and Boland, 1993). Instead of activating protein kinase (PK) C as do phorbol ester tumor promoters, OA is a specific inhibitor of protein phosphatase (PP)1 and PP2A (Holmes and Boland, 1993). PP1 and PP2A dephosphorylate serine and threonine residues in cellular target proteins that are involved in the regulation of multiple signaling pathways (for review, see Wera and Hemmings, 1995). Induction of HO-1 gene expression by activation of PKC (Muraosa and Shibahara, 1993), cAMP-dependent PK (PKA) (Durante et al., 1997; Immenschuh et al., 1998b), or cGMP-dependent PK (PKG) (Immenschuh et al., 1998a) has been demonstrated previously; however, little is known about the role of PPs in the gene regulation of HO-1. Because it has become increasingly obvious that PPs play a major role in maintaining the intracellular balance of gene expression (Hunter, 1995), we investigated the effects of OA on the expression of the HO-1 gene in cultures of primary rat hepatocytes.

In this study, it is shown that OA induces HO-1 gene expression on the protein and mRNA level in a time- and dose-dependent manner. This induction of HO-1 by OA is regulated on the transcriptional level and appears to be mediated by the cAMP response element (CRE)/AP-1 site of the rat HO-1 gene promoter 5′-flanking region.

Experimental Procedures

Animals. Male Wistar rats (2 months old, body weight 170–200 g) were used throughout the study.

Materials. Media M199 and RPMI were obtained from Life Technologies (Karlsruhe, Germany), nitrocellulose filters were from Schleicher and Schuell (Dassel, Germany), and radioisotopes and the chemiluminescent detection system for Western blotting were from Amersham-Buchler (Braunschweig, Germany). The multiprime labeling kit and restriction endonucleases were from New England Biolabs (Cambridge, MA). Falcon tissue culture dishes were from Becton Dickinson (Heidelberg, Germany). OA, calyculin A, and KT5720 were from Calbiochem (San Diego, CA). The polyclonal rabbit anti-rat HO-1 antibody was obtained from Stress Gene (Victoria, Canada). All other chemicals were obtained from Sigma (Deisenhofen, Germany) and Boehringer Mannheim (Mannheim, Germany) unless indicated otherwise.

Cell Isolation and Culture. Hepatocytes were isolated from male Wistar rats by circulating perfusion with collagenase under sterile conditions as described previously (Immenschuh et al., 1998b). The cells were cultured under air/CO_2 (19/1) in medium 199 with Earle's salts containing 2 g/l BSA, 20 mM $NaHCO_3$, 10 mM HEPES, 117 mg/l streptomycin sulfate, 60 mg/l penicillin, 1 nM insulin, and 10 nM dexamethasone. Fetal calf serum (5%) was present during the plating phase up to 4 h, and cell cultures were incubated in serum-free medium for another 18 h before treatment. Hepa 1–6 and NIH3T3 cells were from the American Type Culture Collection (Manassas, VA). Hepa 1–6 cells were cultured in RPMI 1640 medium containing 2% fetal calf serum, and NIH3T3 cells were cultured in DMEM with 10% fetal calf serum until confluency of cell monolayers was reached. Confluent monolayers were incubated in serum-free medium 18 h before treatment.

Tissue macrophages of rat liver (Kupffer cells) were isolated as described (Immenschuh et al., 1999). In brief, the liver was digested with pronase/collagenase solutions, and nonparenchymal cells were separated by density gradient centrifugation. Kupffer cells were purified by counterflow elutriation (J2–21, JE-B6 rotor; Beckmann Instruments, Fullerton, CA), and the obtained Kupffer cells were resuspended in M199 containing 15% fetal calf serum, 100 U of penicillin/ml, and 100 μg of streptomycin/ml. Cell viability was assessed by trypan blue staining, and cells were plated on six-well plates (3×10^6 cells/well). After 2 h, cells were washed for elimination of nonadherent cells, and cell culture was continued with serum-free medium.

Biosynthetic Labeling, Immunoprecipitation, and SDS-Polyacrylamide Gel Electrophoresis (PAGE) of Synthesized Proteins. Hepatocytes were washed with methionine-free M199 and were pulsed for 2 h with M199 containing [^{5}S]methionine (600 μCi/ml). Cell layers were washed with ice-cold PBS, covered with lysis buffer (PBS, 0.5%; deoxycholic acid, 1g%; SDS, 7.4%) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and were frozen at −70°C. After two freezing/thawing cycles in lysis buffer, lysates were centrifuged (10,000g, 30 min, 4°C) and diluted with lysis buffer (1:1). For immunoprecipitation, samples adjusted to contain equal amounts of radioactivity, as determined by trichloroacetic acid precipitation and β-ray counting, were incubated overnight with an excess of antiserum at 4°C. Subsequently, samples were incubated with Pansorbin for 1 h, and the precipitates were washed with lysis buffer and analyzed using SDS-PAGE (15% acrylamide).

Western Blot Analysis. Total protein was prepared from whole liver or cultured hepatocytes by the addition of 1 ml of boiling lysis buffer (0.1% SDS, 10 mM Tris, pH 7.4) and subsequent sonication of liver or scraping of the cells. Cells then were boiled for 5 min and homogenized by passing through a 25-gauge needle. The homogenate was centrifuged for 5 min at 4°C, and the protein content was determined in the supernatant using the Bradford method. Total protein (40 μg) was loaded onto a 10% SDS-polyacrylamide gel and blotted onto nitrocellulose membranes by electrophoresis. Membranes were blocked with Tris-buffered saline containing 1% BSA, 10 mM Tris/HCl (pH 7.5), and 0.1% Tween 20, for 1 h at room temperature. The primary antibody for HO-1 was added in a 1:1000 dilution, and the blot was incubated for 12 h at 4°C. The enhanced chemiluminescent detection system was used for detection.

RNA Isolation, Northern Blot Analysis, and Hybridization. Total RNA for Northern blotting from hepatocytes, Kupffer cells, or whole liver was isolated as described (Immenschuh et al., 1998b, 1999). Equal quantities of RNA were separated on 1.2% agarose, 2.2 M formaldehyde gels. After electrophoresis, RNA was blotted onto nitrocellulose membranes and baked at 80°C for 4 h. After prehybridization for 4 h at 42°C, blots were hybridized overnight with [α-^{32}P]dCTP-radiolabeled cDNA probes at 42°C or a 28S rRNA oligonucleotide as described previously (Immenschuh et al., 1999). The hybridization solution contained 6× standard saline citrate; 5× Denhardt's solution (0.2% Ficoll 400, 0.2% polyvinyl pyrrolidone, and 0.2% BSA); 0.5% SDS; 50% formamide; and 100 μg/ml denatured salmon sperm DNA. Blots were washed subsequently with 2× SSC/0.1% SDS (once) and 0.1× SSC/0.1% SDS (twice) at 65°C. Filters were autoradiographed with X-ray films (X-OMAT RP, Kodak; Rochester, NY) at −70°C for up to 48 h or stored on a phosphorimager screen for 4 to 8 h. Autoradiograms were quantified with phosphorimager running Imagequant software (Molecular Dynamics, Sunnyvale, CA). When nitrocellulose filters were sequentially hybridized with different cDNA probes, the ^{32}P-labeled cDNA was removed after autoradiography by two washing steps with boiling 0.05× SSC/0.1% SDS for 15 min before rehybridization.

cDNA Probes. The probes were the cDNAs of HO-1, phosphoenolpyruvate carboxykinase (PCK), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of rat (Immenschuh et al., 1998b). The cDNAs were labeled by 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 320 mM sucrose, 3 mM $CaCl_2$, 2 mM magnesium acetate, 100 μM EDTA, 100 μM PMSF, 150 μM spermine, 500 μM spermidine, 1 mM dithioerythritol, and 10 mM Tris/HCl, pH 8.0 (buffer A). The cells were scraped off the dishes into buffer A and homogenized in a 2-ml Dounce homogenizer at 4°C. After addi-

tion 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 2 M sucrose, 5 mM magnesium acetate, 100 μM EDTA, 100 μM PMSF, 150 μM spermine, 500 μM spermidine, 1 mM dithioerythritol, and 10 mM Tris/HCl, pH 8.0 (buffer B). This suspension was layered onto a cushion of 2 ml of buffer B and pelleted for 1 h in a Beckman SW60 rotor at 20,000 rpm at 4°C. The pelleted nuclei were suspended in 25 ml of 25% glycerol, 5 mM magnesium acetate, 100 μM EDTA, 100 μM PMSF, 5 mM dithioerythritol, and 50 mM Tris/HCl, pH 8.0 (buffer C).

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 (Immenschuh et al., 1998b). The in vitro transcription reaction was started by the addition of 30 ml of 58% glycerol, 150 mM NH_4Cl, 8.3 mM $MgCl_2$, 830 μM $MnCl_2$, 70 μM EDTA, 25 U of ribonuclease inhibitor, 830 μM ATP, 830 μM CTP, 830 μM GTP, 100 μCi [^{32}P]UTP, and 33 mM HEPES, pH 8.0 (solution D). After incubation of nuclei for 30 min at 37°C, the reaction was stopped by the addition of EDTA.

RNA extraction, prehybridization, and hybridization were performed as described previously (Immenschuh et al., 1998b). In brief, prehybridization was performed in hybridization solution for 12 h at 42°C, followed by hybridization for 72 h at 42°C, 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 NaCl/sodium citrate solution.

Plasmid Constructs. The rat HO-1 promoter 5′-flanking region from −1338 to +71 was amplified by PCR from rat genomic DNA by using the oligonucleotide 5′-CTCAGGATTAACAAAACAAAGACACAAAAAG-3′ (−1338/−1309) as forward and 5′-GAGATGGCTCTGCTCCGGCAGGCTCCACTC-3′ (+42/+71) as reverse primer, respectively. The resulting PCR product was blunted by Klenow enzyme and phosphorylated with T4 polynucleotide kinase and ligated into the *Sma*I site of pUC18. The insert was excised with *Kpn*I/*Bam*HI and cloned into the *Kpn*I/*Bgl*II site of pGl3basic (Promega, Madison, WI) (pHO-1338 Luc; see Fig. 7, construct 1). Construction of plasmid pHO-754 Luc (see Fig. 7, construct 2), mutated rat HO-1 gene promoter constructs (see Fig. 7, pHO-754del, construct 3; and pHOΔCRE/AP-1, construct 4), and chloramphenicol acetyltransferase (CAT) construct pPCK-2500 CAT has been described previously in detail (Immenschuh et al., 1998a, Bratke et al., 1999). All constructs were verified by sequencing in both directions.

Cell Transfection, Luciferase, and CAT Assay. Rat hepatocyte cultures (~1×10^6 cells per dish) were transfected transiently with 2.5 μg of plasmid DNA containing 500 ng of pRL-SV40 (Promega) to control transfection efficiency and 2 μg of the HO-1 promoter luciferase construct (Immenschuh et al., 1998a). Luciferase and CAT activity were determined as described previously (Immenschuh et al., 1998a; Bratke et al., 1999).

PCK Enzyme Activity. PCK enzyme activity was determined in duplicate as described previously (Bratke et al., 1999).

Results

OA-Dependent Induction of HO-1 Gene Expression in Cultures of Primary Rat Hepatocytes. To study the effect of the PP inhibitor OA on the synthesis of HO-1, newly synthesized proteins were pulse-labeled with [^{35}S]methionine in primary rat hepatocytes treated with OA at various concentrations, and HO-1 protein was immunoprecipitated from cell lysates. As shown in Fig. 1A, HO-1 protein was dose dependently up-regulated in the presence of OA. Next, we determined the effect of OA on steady-state levels of HO-1 mRNA. HO-1 message was markedly induced in hepatocyte cultures after 6 h (Fig. 1B). For comparison, no up-regulation of HO-1 mRNA expression by OA was observed in cultured rat liver tissue macrophages (Kupffer cells; Fig. 1C). The induction of HO-1 mRNA expression by heme, which is one of the most effective inducers of this enzyme, is shown as a positive control in Kupffer cells (Fig. 1C, lane 4). In two hepatoma cell lines (H35 and Hepa 1-6) and NIH3T3 fibroblasts, treatment with OA had no effect on HO-1 mRNA steady-state levels (data not shown). Because HO-1 activity has been shown to be increased during the first days of cell culture of primary rat hepatocytes (Schuetz et al., 1988), we compared HO-1 gene expression in our system of hepatocyte cultures with that in whole liver. Both the expression of HO-1 protein and mRNA were higher in 24-h cultured rat hepato-

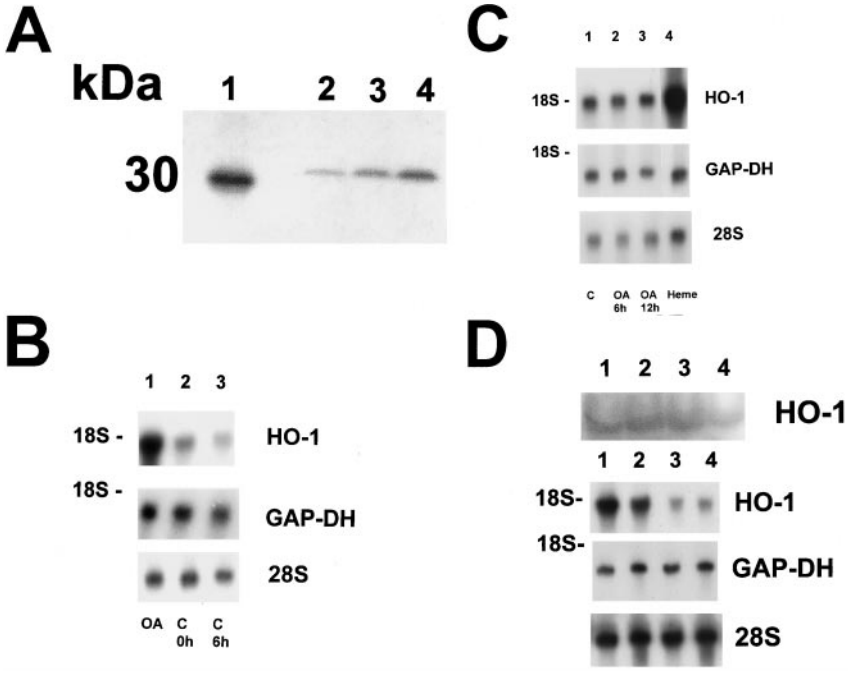

Fig. 1. Up-regulation of HO-1 gene expression in cultured primary rat hepatocytes, but not in rat liver tissue macrophages (Kupffer cells). Primary rat hepatocytes and Kupffer cells were isolated and cultured as described in *Experimental Procedures*. A, hepatocytes were treated for 18 h with serum-free medium before cell culture was continued under control conditions (lane 2) or in the presence of OA at concentrations of 5 nM (lane 3) and 10 nM (lane 4). Newly synthesized proteins were pulse-labeled with [^{35}S]methionine for 16 h, and HO-1 protein was immunoprecipitated from cell lysates. Immunoprecipitates were analyzed using SDS-PAGE and autoradiography. The size marker is shown in lane 1. After overnight culture in serum-free medium, (B) hepatocytes (0 h, lane 2) were cultured for 6 h under control conditions (lane 3) or in the presence of OA (10 nM; lane 1), and (C) Kupffer cells were cultured under control conditions for 6 h (lane 1) or in the presence of OA (10 nM) for 6 h (lane 2) and 12 h (lane 3) or with heme for 6 h (10 μM; lane 4). Total cellular RNA (15 μg) was assessed by Northern blot analysis and sequentially probed with the ^{32}P-labeled cDNAs of HO-1 and GAPDH and a 28S rRNA oligonucleotide. The size marker was the 18S ribosomal RNA band. D, protein (top panel) and total RNA (bottom panel) from primary rat hepatocytes after 24 h (lane 1), 48 h (lane 2), and 72 h in cell culture (lane 3), and from whole liver (lane 4) were assessed by Northern and Western blot analysis as described in *Experimental Procedures*. Autoradiograms from representative experiments are shown in A to D, respectively.

cytes compared with that in whole liver (Fig. 1D). Thereafter, HO-1 mRNA and protein declined and reached approximately the level of whole liver after 72 h of cell culture. To exclude the possibility that OA augments the effect of a stimulating factor, which may be generated during the isolation of rat hepatocytes, rather than stimulating the HO-1 gene expression per se, hepatocytes also were treated with OA after 120 h of cell culture. The OA-dependent induction of HO-1 gene expression in these long-term cultured hepatocytes was 14- ± 1.5-fold (n = 3) on the mRNA level and 4- ± 0.6-fold (n = 3) on the protein level (data not shown).

The increase of HO-1 mRNA expression after exposure to OA was time-dependent, reaching a transient maximum after 6 h (Fig. 2A). Moreover, the induction of HO-1 by OA was dose-dependent, with a peak at 10 nM. For normalization, the mRNA expression of GAPDH and that of the 28S rRNA were determined. Because GAPDH mRNA was not appreciably affected by the treatment with OA (Fig. 2, A and B; see also Fig. 1, B–D), in the following, GAPDH was used for normalization of HO-1 mRNA expression. The range of OA concentrations regulating HO-1 gene expression was similar to that observed for the mRNA regulation of the cytochrome P450 (CYP) isozymes 2B1 and 2B2 in primary rat hepatocyte cultures (Sidhu and Omiecinski, 1997). At OA concentrations >50 nM, hepatocytes were damaged severely as observed by light microscopy and lactate dehydrogenase leakage (data not shown). For a quantitative comparison, the effects of heme (10 μM), $CoCl_2$ (100 μM), and dibutyryl cAMP

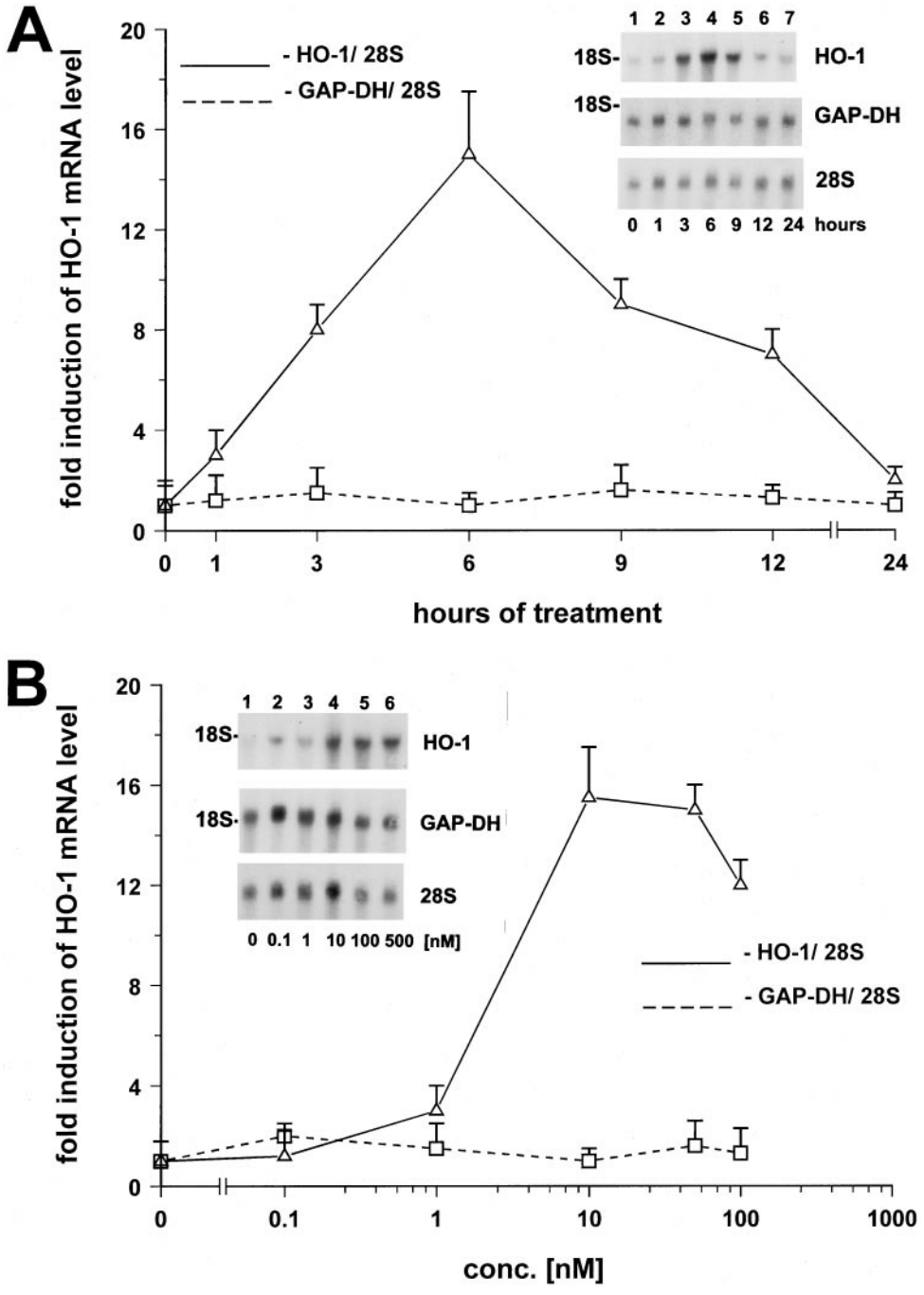

Fig. 2. Time- and dose-dependent induction of HO-1 mRNA expression by OA in rat hepatocyte cultures. Primary rat hepatocytes were isolated and cultured as described in *Experimental Procedures*. Hepatocytes were treated for 18 h with serum-free medium before cell culture was continued (A) in the presence of OA (10 nM; lanes 2–7) for the times indicated, or (B) for 6 h in the absence (lane 1) or presence of increasing concentrations of OA (0.1–100 nM; lanes 2–6). Total cellular RNA (15 μg) was assessed by Northern blot analysis and sequentially probed with the ^{32}P-labeled cDNAs of HO-1 and GAPDH or a 28S rRNA oligonucleotide. The size marker was the 18S ribosomal RNA band. Autoradiograms were quantitated by densitometry, and values represent the fold induction rate of HO-1 mRNA normalized to the 28S rRNA signal or that of the GAPDH mRNA normalized to the 28S rRNA signal from at least three independent experiments (mean ± S.E.).

(Bt_2cAMP; 250 μM) on HO-1 mRNA expression in rat hepatocyte cultures are shown in Table 1. We also examined HO-1 mRNA expression in the presence of calyculin A, which has a lower inhibitory effect on PP2A, but a higher inhibitory effect on PP1 compared with OA (Holmes and Boland, 1993). In contrast to OA, calyculin A had no effect on steady-state levels of HO-1 mRNA (Table 1); however, it exhibited cell toxicity similar to that in OA. Comparable with these findings, a diverging effect of OA and calyculin A has been demonstrated previously for the regulation of nerve growth factor expression in primary rat astrocyte cultures (Pshenichkin and Wise, 1995).

From the data, we conclude that HO-1 gene expression is induced in a time- and dose-dependent manner by OA in primary cultures of rat hepatocytes.

Differential Effects of OA on the Bt_2cAMP-Dependent Induction of HO-1 and PCK Gene Expression. Because HO-1 gene expression is induced by activation of PKA in primary rat hepatocyte cultures (Immenschuh et al., 1998b) and OA has been shown to augment the transcriptional response to cAMP (Hagiwara et al., 1992), hepatocytes were treated with the specific inhibitor of PKA, KT5720, before OA was added for another 6 h. As shown in Fig. 3A, pretreatment with KT5720 reduced the OA-dependent HO-1 mRNA induction by >50%. Moreover, simultaneous treatment of hepatocytes with Bt_2cAMP and OA at submaximal doses caused a synergistic induction of HO-1 mRNA expression (Fig. 3B). To investigate the putative cross-talk of OA with the PKA signaling pathway, we also examined the effect of OA on the expression of the PCK gene, which is a liver-specific, cAMP-induced gene. PCK catalyzes the rate-controlling step of the gluconeogenic pathway and is induced by a variety of stimuli enhancing intracellular cAMP levels (for review, see Hanson and Reshef, 1997). In contrast to the OA-dependent regulation of HO-1 gene expression, OA on its own did not affect basal PCK mRNA expression or enzyme activity (Fig. 4). Simultaneous treatment of Bt_2cAMP-treated hepatocytes with OA reduced the PCK mRNA expression and enzyme activity elicited by Bt_2cAMP dose dependently (Fig. 4). These findings on the PCK gene expression are in agreement with those from a previous report in H4IIE rat hepatoma cells (O'Brien et al., 1994). Therefore, OA may affect the PKA signaling pathway in primary rat hepatocytes; however, it may result in differential regulation of cAMP-activated gene expression.

TABLE 1

Comparative effects of treatment with OA, calyculin A, heme, $CoCl_2$, and Bt_2cAMP on HO-1 mRNA expression in primary cultures of rat hepatocytes

Rat hepatocytes were treated for 18 h with serum-free medium before cell culture was continued in the presence of various agents for 6 or 12 h. Total RNA was isolated as described in *Experimental Procedures* and assessed by Northern blot analysis. Values show the fold induction rate relative to the basal HO-1 mRNA expression at 0 h normalized to the GAPDH mRNA levels of at least three independent experiments (mean ± S.E.). Student's *t* test for paired values: *indicates significant differences control versus OA, control versus heme, control versus $CoCl_2$, and control versus Bt_2cAMP, $P \leq .05$.

Treatment	Fold induction of HO-1 mRNA	
	6 h	12 h
Control	1 ± 0.2	1 ± 0.3
OA (10 nM)	13 ± 1.5*	7 ± 1*
Calyculin A (1 nM)	1 ± 0.2	1 ± 0.2
Calyculin A (20 nM)	0.9 ± 0.1	1 ± 0.1
Heme (10 μM)	40 ± 5*	15 ± 4*
$CoCl_2$ (100 μM)	45 ± 6*	18 ± 2.5*
Bt_2cAMP (250 μM)	19 ± 2*	7 ± 2*

Transcriptional Induction of HO-1 Gene Expression by OA. Up-regulation of the HO-1 gene occurs on the transcriptional level by most stimuli (Shibahara et al., 1987; Choi and Alam, 1996; Durante et al., 1997; Immenschuh et al., 1998b). To probe into the mechanism of the OA-dependent HO-1 gene induction, hepatocyte cultures were treated with the transcription inhibitor actinomycin D (ActD) and the protein synthesis inhibitor cycloheximide (CHX). Both agents were added at a concentration of 1 μg/ml for 30 min before OA was added for another 6 h. Neither ActD nor CHX alone had an effect on the basal HO-1 mRNA expression, respectively (Fig. 5A). ActD prevented the OA-dependent HO-1 mRNA induction. CHX reduced the OA-elicited HO-1 mRNA expression levels by 50% (Fig. 5A). Because the data indicated a transcriptional mode of induction, nuclear run-off assays were performed with nuclei from OA-treated hepatocyte cultures. The transcription rate of the HO-1 gene was

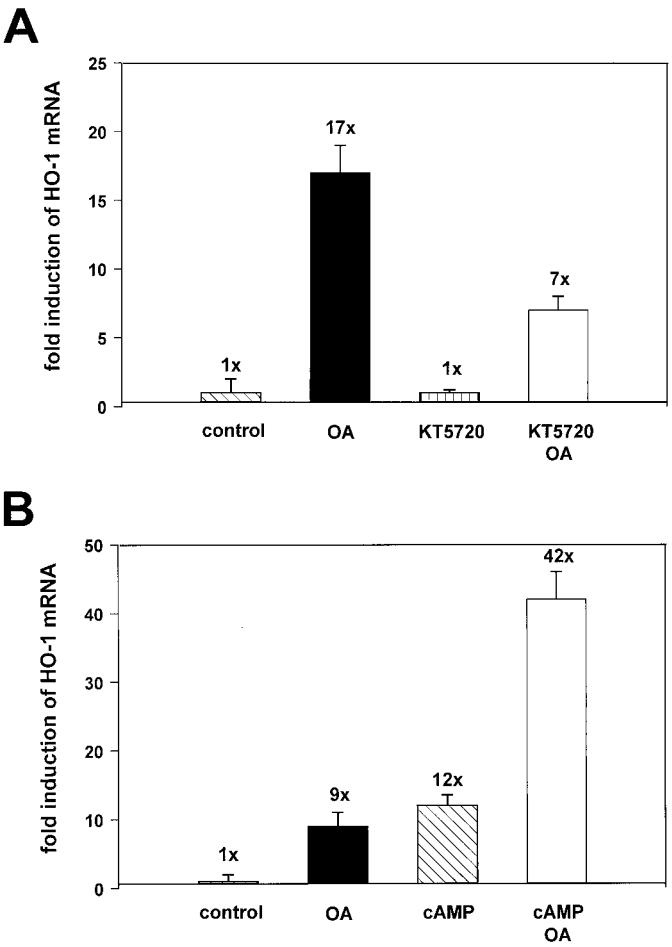

Fig. 3. Inhibition of the OA-dependent HO-1 mRNA induction by KT5720 and synergistic induction of HO-1 mRNA by OA and Bt_2cAMP in rat hepatocyte cultures. Hepatocytes were cultured as described in *Experimental Procedures*. After 18 h in serum-free medium, cell culture was continued (A) in the presence of OA (10 nM), KT5720 (1 μM), and a combination of OA plus KT5720 for 6 h, or (B) in the presence of OA (5 nM), Bt_2cAMP (100 μM), and a combination of OA plus Bt_2cAMP for 6 h. Total RNA was assessed by Northern blot analysis and was sequentially probed with the ^{32}P-labeled cDNAs of HO-1 and GAPDH. Autoradiograms were quantitated, and values represent the fold induction rate of HO-1 mRNA normalized to GAPDH from at least three independent experiments (mean ± S.E.).

strongly increased by OA (Fig. 5B). The turnover rate of HO-1 mRNA was determined in cell cultures after exposure to OA. As shown in Fig. 6, the half-life of HO-1 mRNA was slightly decreased during treatment with OA (4.7 h versus 4.2 h).

The data show that the induction of HO-1 gene expression by OA is primarily regulated on the transcriptional level and that de novo protein synthesis contributes partially to the HO-1 gene activation by OA in hepatocyte cultures.

OA-Dependent Induction of the Rat HO-1 Gene Promoter in Transiently Transfected Rat Hepatocyte Cultures. To investigate whether regulatory elements of the rat HO-1 5′-flanking promoter region are involved in the transcriptional regulation by OA, luciferase reporter constructs containing either the proximal 1338 or the 754 base pairs of the rat HO-1 promoter region were transiently transfected into primary rat hepatocyte cultures (Fig. 7, constructs 1 and 2; Table 2, pHO-1338 Luc and pHO-754 Luc). OA up-regulated the luciferase expression of these constructs 4- and 5.5-fold, respectively (Fig. 7), and a combination of submaximal doses of OA plus Bt_2cAMP induced luciferase expression additively (Table 2). An HO-1 reporter construct with a deletion from −714 to −549 (Fig. 7, construct 3) and a construct lacking the CRE/AP-1 site (Fig. 7, construct 4) were not regulated by OA (Fig. 7). For a comparison, the regulation of a CAT reporter construct containing 2500 base pairs of the rat PCK promoter 5′-flanking region was examined in transfected rat hepatocytes (Table 2). Whereas treatment with OA alone had no effect, the Bt_2cAMP-dependent induction of this reporter construct was inhibited by OA (Table 2, pPCK-2500 CAT).

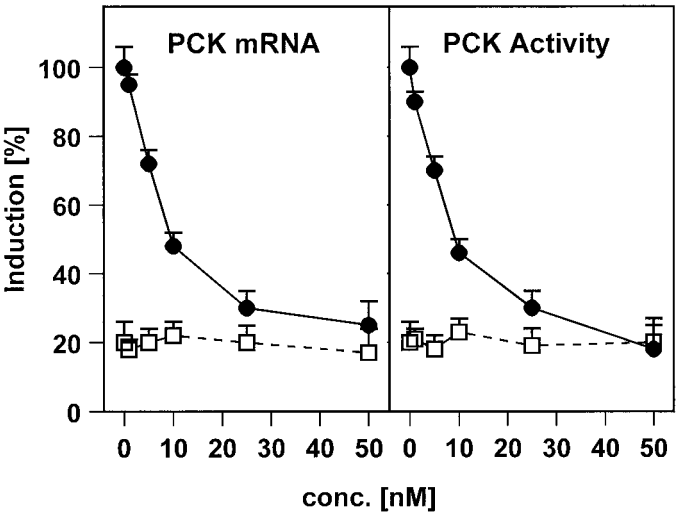

Fig. 4. Down-regulation of the Bt_2cAMP-dependent induction of PCK mRNA expression and enzyme activity by OA in rat hepatocyte cultures. Hepatocytes were cultured as described in *Experimental Procedures*. Left, cells were cultured in the absence (□) or presence of Bt_2cAMP (250 μM; ●) for 2 h until the maximal PCK mRNA induction was reached with OA at increasing concentrations. Total RNA was isolated and subjected to Northern blot analysis. Blots were sequentially probed with the ^{32}P-labeled cDNAs of PCK and GAPDH. Autoradiograms were quantitated, and values shown represent the PCK mRNA levels relative to the mRNA expression after treatment with Bt_2cAMP alone from at least three independent experiments (mean ± S.E.). Right, hepatocytes were cultured for 4 h in the absence (□) or presence of Bt_2cAMP (250 μM; ●) with OA at increasing concentrations. Then PCK enzyme activity was determined. Values are from at least three independent experiments and represent the PCK enzyme activity relative to that after treatment with Bt_2cAMP alone (mean ± S.E.).

The data indicate that the CRE/AP-1 site is involved in the HO-1 gene regulation by OA and that the differential regulation of the HO-1 and PCK gene promoters by OA and Bt_2cAMP is similar to that of the OA-dependent regulation of the endogenous HO-1 and PCK genes in rat hepatocyte cultures.

Discussion

In this study, it is shown in cultured rat hepatocytes that the serine threonine PP inhibitor OA up-regulates the gene expression of HO-1, which is the inducible enzyme of heme degradation. The OA-dependent HO-1 induction occurs on the transcriptional level and is mediated by a DNA sequence of the HO-1 gene promoter 5′-flanking region.

The OA-dependent increase of HO-1 gene expression is primarily regulated on the transcriptional level, as demon-

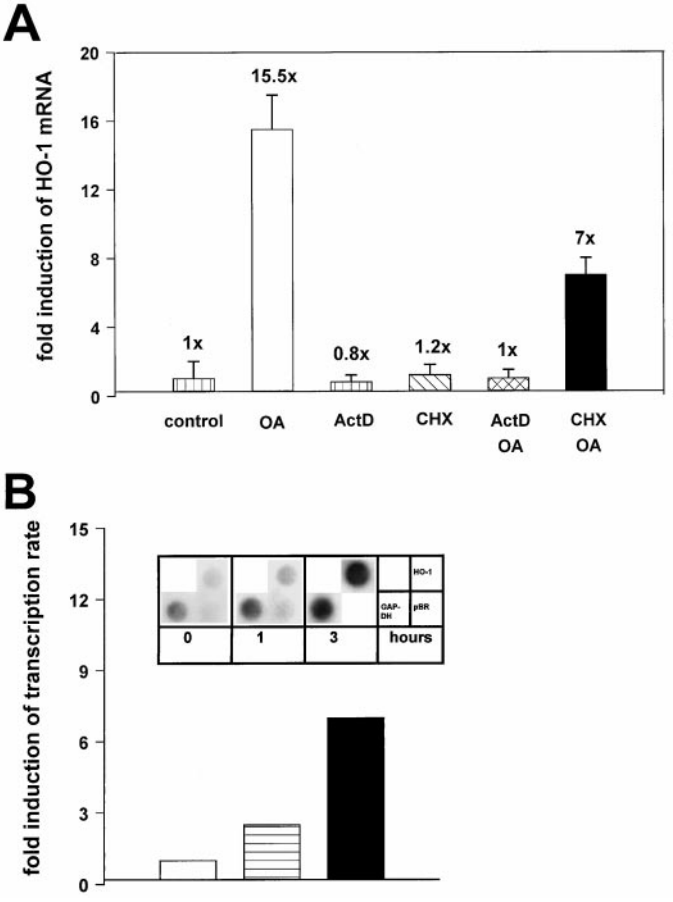

Fig. 5. Transcriptional regulation of the OA-dependent gene activation. Inhibition by ActD and CHX of OA-dependent induction of HO-1 mRNA expression. A, hepatocytes were pretreated for 30 min with ActD (1 μg/ml) or CHX (1 μg/ml), and OA (10 nM) was added for another 6 h, after which total RNA was isolated and assessed by Northern blot analysis. The blots were probed sequentially with the ^{32}P-labeled cDNAs of HO-1 and GAPDH. Values given represent the fold induction rate of HO-1 mRNA normalized to GAPDH levels from two or three independent experiments (mean ± S.E.). B, after 18 h in serum-free medium, hepatocyte culture was continued in the presence of OA (10 nM). At 0, 1, and 3 h, nuclei were prepared and subjected to nuclear run-off transcription assay as described in *Experimental Procedures*. Radiolabeled nascent RNA transcripts were purified and hybridized to HO-1 and GAPDH cDNAs or pBR322 immobilized on nitrocellulose paper, as indicated. The plasmid pBR322 was used as a control for nonspecific hybridization. Autoradiograms were quantitated, and bar graphs represent the fold induction rate of HO-1 mRNA normalized to GAPDH from a representative experiment.

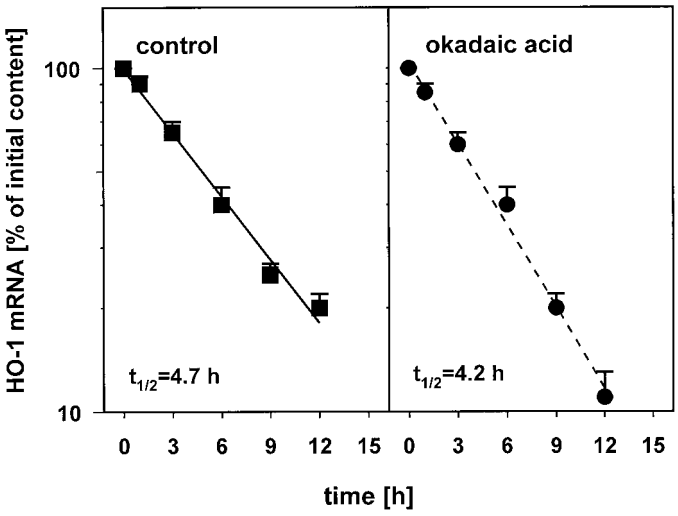

Fig. 6. Effect of OA on the half-life of HO-1 mRNA in rat hepatocyte cultures. Rat hepatocytes were cultured as described in *Experimental Procedures*. Hepatocytes were cultured either in the absence (left) or presence of OA (right) (10 nM) for 6 h, after which cell culture was continued with ActD (1 μg/ml). Total RNA was isolated at the times indicated, and the levels of HO-1 mRNA were determined by Northern blot analysis. The primary plot is a semilog plot of individual points of two independent experiments (mean ± S.E.). The half-lives calculated from the graphs are indicated, respectively.

strated by blocking of the HO-1 mRNA induction with ActD (Fig. 5A), nuclear run-off assay (Fig. 5B), and transfection of HO-1 reporter gene constructs into hepatocyte cultures (Fig. 7). Stabilization of HO-1 message is not involved in the OA-dependent HO-1 gene regulation (Fig. 6), but protein synthesis de novo appears to contribute to this induction, as indicated by the inhibition of OA-dependent HO-1 mRNA expression by CHX (Fig. 5A) suggesting that protein(s) with a short half-life participate(s) in this regulatory pathway. Therefore, OA adds to the various stimuli that modulate the transcription rate of the HO-1 gene (Shibahara et al., 1987; Applegate et al., 1991; Durante et al., 1997; for review, see Choi and Alam, 1996). Several REs within the promoter 5′-flanking region that are involved in the activation of the human, mouse, and rat HO-1 genes have been characterized (for review, see Choi and Alam, 1996). As demonstrated by transient transfection of rat HO-1 gene reporter constructs into rat hepatocytes, the CRE/AP-1 element of the rat HO-1 gene (position −665 to −654) (Immenschuh et al., 1998a) is involved in the OA-dependent regulation of the HO-1 gene (Fig. 7). Although deletion of the HO-1 CRE/AP-1 element abolished the OA-dependent induction of luciferase reporter gene activity, it cannot be excluded that additional REs are involved in the OA-dependent gene regulation. A potential transcription factor (TF) that may mediate the OA-dependent transcriptional induction is the CRE-binding protein (CREB), which is activated on phosphorylation at Ser-133. Hagiwara et al. (1992) have demonstrated that OA inhibits the dephosphorylation of the Ser-133 of phospho-CREB, thereby augmenting cAMP-dependent gene expression. The hypothesis that CREB may mediate the OA-dependent HO-1 induction is supported by the observations that OA and Bt_2cAMP elicit a synergistic effect on HO-1 mRNA up-regulation and that the specific PKA inhibitor KT5720 reduces the induction of HO-1 mRNA expression by OA (Fig. 3). Moreover, the pHO-1338 Luc and pHO-754 Luc HO-1 gene reporter constructs are up-regulated additively by submaximal doses of OA and Bt_2cAMP (Table 2). Whether the Ser-133 of CREB is dephosphorylated by PP1 or PP2A appears to be cell type-dependent. Alberts et al. (1994) have shown that PP1 is the major regulator of dephosphorylation of CREB in fibroblasts. By contrast, others have demonstrated in rat liver and HepG2 hepatoma cells that PP2A dephosphorylates phospho-CREB 30-fold more efficiently than does PP1 (Wadzinski et al., 1993). The latter finding would correlate with our observation that OA, but not calyculin A, induced HO-1 gene expression in rat hepatocyte cultures at the applied concentrations (Table 1). OA has been reported to inhibit PP2A ~5- to 10-fold stronger than does calyculin A, whereas calyculin A is a significantly stronger inhibitor of PP1 than OA (Holmes and Boland, 1993). In contradiction to the idea that CREB may mediate the OA-dependent HO-1 induction on its own is the inhibitory effect of OA on the cAMP-dependent PCK gene expression. The cAMP-dependent induction of the PCK gene is known to be primarily mediated via a CRE (Hanson and Reshef, 1997); however, in our system of primary rat hepatocyte cultures, the cAMP-dependent induction of the PCK gene was inhibited by OA (Fig. 4, Table 2), as similarly reported in H4IIE hepatoma cells (O'Brien et al., 1994). It also has been shown that the liver-specific induction of the PCK gene promoter requires synergism of the TFs CREB and C/EBPα to mediate the full cAMP response in hepatic cells (Roesler et al., 1996). Other

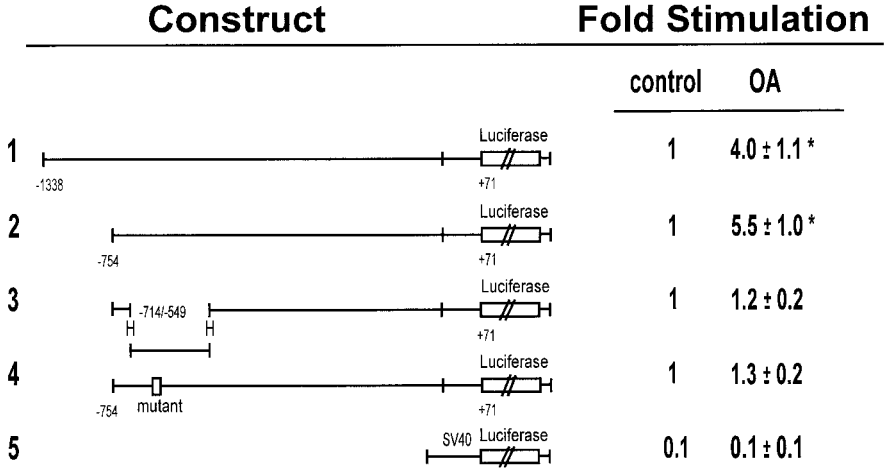

Fig. 7. OA-dependent regulation of DNA sequences of the rat HO-1 gene promoter 5′-flanking region in transiently transfected rat hepatocyte cultures. The indicated rat HO-1 gene sequences were cloned into pGL3Luc (constructs 1–4) as described in *Experimental Procedures*. The reporter constructs were transiently transfected into primary rat hepatocyte cultures and, after 24 h, the transfected cells were treated for 12 h with OA (10 nM). The rate of induction in each experiment relative to the control was determined. Regulation of luciferase activity of pGL3prom is shown as a control. The values are from at least three independent experiments (mean ± S.E.). Student's *t* test for paired values: * indicates significant differences control versus OA, $P \leq .05$. In construct 3, H indicates *Hin*dIII restriction site.

TFs that have been demonstrated to be involved in the OA-dependent gene expression are NFκB and AP-1. NFκB has been reported to be activated by OA via phosphorylation and subsequent degradation of IκB (Sun et al., 1995), and increased binding of AP-1 to its recognition sequence by OA has been demonstrated in Syrian hamster hepatocytes (Tohkin et al., 1996) and in a mouse keratinocyte cell line (Rosenberger and Bowden, 1996).

What are the signaling pathways that are involved in the HO-1 gene regulation by OA? HO-1 mRNA induction by OA was observed in rat hepatocytes, but not in cell cultures such as liver tissue macrophages (Fig. 1C), which exhibit a high basal level of HO-1 gene expression (Bauer et al., 1998; Immenschuh et al., 1999), or in NIH3T3 fibroblasts (data not shown), suggesting a hepatocyte-specific signaling pathway. The data correspond with a previous study showing that the PKA-dependent induction of HO-1 is specific in primary rat hepatocyte cultures (Immenschuh et al., 1998b). Similar findings have been reported for the inducible nitric-oxide synthase (iNOS) by Pahan et al. (1998), who have demonstrated contrasting effects of OA on the expression of iNOS in rat astrocyte and macrophage cell cultures. Because PP2A is known to deactivate the extracellular signal-regulated kinases (ERK) 1/2 (Hunter, 1995), it is conceivable that the inhibition of PP2A by OA may activate these mitogen-activated PKs. In fact, it has been shown recently that ERK 1/2 participate in the OA-dependent transcriptional induction of the human collagenase gene via AP-1 activation in mouse keratinocytes (Rosenberger et al., 1999). As to the role of ERKs in the induction of HO-1 gene expression by stress inducers, the available data are not conclusive. Elbirt et al. (1998) have reported that for chicken HO-1 gene promoter constructs in transiently transfected LMH chicken hepatoma cells, ERKs may be involved in the regulation of HO-1 by sodium arsenite. By contrast, Masuya et al. (1998) have demonstrated that for the endogenous human HO-1 gene expression in HeLa cells, tyrosine kinases rather than mitogen-activated kinases, are involved in the regulation of HO-1 gene expression by various stress inducers including sodium arsenite.

Because the cellular "free heme pool" of hepatocytes, e.g., the nonprotein bound portion of heme in hepatocytes (Granick et al., 1975), is regulated via the enzymatic degradation by HO, the OA-dependent induction of HO-1 expression may significantly decrease the cellular heme availability in hepatocytes. A low "free heme pool," in turn, could decrease the enzyme activity of the iNOS. Albakri and Stuehr (1996) have demonstrated that sufficient intracellular heme is essential for the formation of dimeric iNOS and its catalytic activity. HO-1 is thought to provide protection against oxidative stress, most likely attributable to the fact that HO enzymatically degrades the pro-oxidant heme leading to the formation of the antioxidant bilirubin (Stocker et al., 1987). This assumption is underscored by findings that HO-1 deficient mice are highly susceptible to the toxic effects of oxidative stress (Poss and Tonegawa, 1997b). Recently, the first case of human HO-1 deficiency has been described (Yachie et al., 1999), showing characteristics similar to those observed in HO-1-deficient mice (Poss and Tonegawa, 1997b). The induction of HO-1 by the PP inhibitor OA indicates that the balance between cellular kinases and phosphatases is important for the regulation of HO-1 gene expression. Additional studies to elucidate the detailed regulatory pathways of HO-1 gene expression are necessary to develop strategies for a potential targeted pharmacologic modulation of HO-1.

TABLE 2

Regulation of DNA sequences of the rat HO-1 and PCK gene promoter 5′-flanking regions by OA and Bt_2cAMP in transiently transfected rat hepatocyte cultures

The indicated rat HO-1 or PCK gene sequences were cloned into pGL3Luc or pCAT, as indicated, and the reporter constructs were transiently transfected into primary rat hepatocyte cultures. After 24 h, the transfected cells were treated for 12 h with OA (5 nM), Bt_2cAMP (100 μM) or a combination of OA plus Bt_2cAMP. The rate of induction in each experiment relative to the control was determined. The values are from three independent experiments (mean ± S.E.). Student's t test for paired values: *indicates significant difference OA versus OA + Bt_2cAMP; $P \leq .05$; **significant difference OA versus Bt_2cAMP, $P \leq .05$.

Construct	Fold induction of reporter gene activity		
	OA	Bt_2cAMP	OA + Bt_2cAMP
pHO-1338 Luc	2.5 ± 0.2	2.5 ± 0.3	4.5 ± 0.5*
pHO-754 Luc	3 ± 0.5	3.5 ± 1	6 ± 0.4*
pPCK-2500 CAT	1 ± 0.2	4 ± 0.4**	2.5 ± 0.2
pGL3prom Luc	0.1 ± 0.2	0.1 ± 0.1	0.1 ± 0.2

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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

Albakri QA and Stuehr DJ (1996) Intracellular assembly of inducible NO synthase is limited by nitric oxide-mediated changes in heme insertion and availability. *J Biol Chem* **271:**5414–5421

Alberts AS, Montminy M, Shenolikar S and Feramisco JR (1994) Expression of a peptide inhibitor of protein phosphatase 1 increases phosphorylation and activity of CREB in NIH3T3 fibroblasts. *Mol Cell Biol* **14:**4398–4407

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

Bauer I, Wanner GA, Rensing H, Alte C, Miescher E, Wolf B, Pannen BHJ, Clemens MG and Bauer M (1998) Expression pattern of heme oxygenase isoenzymes 1 and 2 in normal and stress-exposed rat liver. *Hepatology* **27:**829–838

Bratke J, Kietzmann T and Jungermann K (1999) Identification of an oxygen responsive element in the 5′-flanking sequence of the rat cytosolic phosphoenolpyruvate carboxykinase 1 gene modulating its glucagon-dependent activation. *Biochem J* **339:**563–569

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

Elbirt KK, Whitmarsh AJ, Davis RJ and Bonkovsky HL (1998) Mechanism of sodium arsenite-mediated induction of heme oxygenase-1 in hepatoma cells. Role of mitogen-activated protein kinases. *J Biol Chem* **273:**8922–8931

Granick S, Sinclair P, Sassa S and Grieninger G (1975) Effects by heme, insulin, and serum albumin on heme and protein synthesis in chick embryo liver cells cultured in a chemically defined medium, and a spectrofluorometric assay for porphyrin composition. *J Biol Chem* **250:**9215–9225

Hagiwara M, Alberts A, Brindle P, Meinkoth J, Feramisco J, Deng T, Karin M, Shenolikar S and Montminy M (1992) Transcriptional attenuation following cAMP induction requires PP-1-mediated dephosphorylation of CREB. *Cell* **70:**105–113

Hanson RW and Reshef L (1997) Regulation of the phosphoenolpyruvate carboxykinase (GTP) gene expression. *Annu Rev Biochem* **66:**581–611

Holmes CFB and Boland MP (1993) Inhibitors of protein phosphatase-1 and -2A; two of the major serine/threonine protein phosphatases involved in cellular regulation. *Curr Opin Struct Biol* **3:**934–943

Hunter T (1995) Protein kinases and phosphatases: The yin and yang of protein phosphorylation and signaling. *Cell* **80:**225–236

Immenschuh S, Hinke V, Ohlmann A, Gifhorn-Katz S, Katz N, Jungermann K and Kietzmann T (1998a) Transcriptional activation of the heme oxygenase-1 gene by cGMP via a cAMP response element/activator protein-1 element in primary rat hepatocytes. *Biochem J* **334:**141–146

Immenschuh S, Kietzmann T, Hinke V, Wiederhold M, Katz N and Muller-Eberhard U (1998b) The rat heme oxygenase-1 gene is transcriptionally induced via the protein kinase A signaling pathway in rat hepatocyte cultures. *Mol Pharmacol* **53:**483–491

Immenschuh S, Tan M and Ramadori G (1999) Nitric oxide mediates the lipopolysaccharide dependent upregulation of the heme oxygenase-1 gene expression in cultured rat Kupffer cells. *J Hepatol* **30:**61–69
Lee PJ, Alam J, Wiegand GW and Choi AMK (1996) Overexpression of heme oxygenase-1 in pulmonary epithelial cells results in cell growth arrest and increased resistance to hyperoxia. *Proc Natl Acad Sci USA* **93:**10393–10398
Maines M (1988) Heme oxygenase: Function, multiplicity, regulatory mechanisms, and clinical applications. *FASEB J* **2:**2557–2568
Maines MD, Trakshel GM and Kutty RK (1986) Characterization of two constitutive forms of rat liver microsomal heme oxygenase. Only one molecular species of the enzyme is inducible. *J Biol Chem* **261:**411–419
Masuya Y, Hioki K, Tokunaga R and Taketani S (1998) Involvement of the tyrosine phosphorylation pathway in induction of human heme oxygenase-1 by hemin, sodium arsenite, and cadmium chloride. *J Biochem* **124:** 628–633
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
O'Brien RM, Noisin E and Granner DK (1994) Comparison of the effects of insulin and okadaic acid on phosphoenolpyruvate carboxykinase gene expression. *Biochem J* **303:**737–742
Pahan K, Sheikh FG, Namboodiri AM and Singh I (1998) Inhibitors of protein phosphatase 1 and 2A differentially regulate the expression of inducible nitric-oxide synthase in rat astrocytes and macrophages. *J Biol Chem* **273:**12219–12226
Poss KD and Tonegawa S (1997a) Heme oxygenase is required for mammalian iron reutilization. *Proc Natl Acad Sci USA* **94:**10925–10930
Poss KD and Tonegawa S (1997b) Reduced stress defense in heme oxygenase 1-deficient cells. *Proc Natl Acad Sci USA* **94:**10925–10930
Pshenichkin SP and Wise BC (1995) Okadaic acid increases nerve growth factor secretion, mRNA stability, and gene transcription in primary cultures of cortical astrocytes. *J Biol Chem* **270:**5994–5999
Roesler WJ, Crosson SM, Vinson C and McFie PJ (1996) The α-isoform of the CCAAT/enhancer-binding protein is required for mediating cAMP responsiveness of the phosphoenolpyruvate carboxykinase promoter in hepatoma cells. *J Biol Chem* **271:**8068–8074
Rosenberger SF and Bowden GT (1996) Okadaic acid stimulated TRE binding activity in a papilloma producing mouse keratinocyte cell line involves increased AP-1 expression. *Oncogene* **12:**2301–2308
Rosenberger SF, Finch JS, Gupta A and Bowden GT (1999) Extracellular signal-regulate kinase 1/2-mediated phosphorylation of JunD and FosB is required for okadaic acid-induced activator protein 1 activation *J Biol Chem* **274:**1124–1130
Schuetz EG, Li D, Omiecinski CJ, Muller-Eberhard U, Kleinman HK Elswick B and Guzelian PS (1988) Regulation of gene expression in adult rat hepatocytes cultured on a basement membrane matrix. *J Cell Physiol* **134:**309–323.
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 (1997) An okadaic acid-sensitive pathway involved in the phenobarbital-mediated induction of CYP2B gene expression in primary rat hepatocyte cultures. *J Pharmacol Exp Ther* **282:**1122–1129
Stocker R, Yamamoto Y, Mc Donagh AN, Glazer AN and Ames BN (1987) Bilirubin is an antioxidant of possible physiological significance. *Science* **235:**1043–1046
Sun SC, Maggirwar SB and Harhaj E (1995) Activation of NF-κB by phosphatase inhibitors involves the phosphorylation of IκB α at phosphatase 2A-sensitive sites. *J Biol Chem* **270:**18347–18351
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
Tohkin M, Kurose K and Fukuhara M (1996) Okadaic acid potentiates 3-methylcholanthrene-induced CYP2A8 gene expression in primary cultures of syrian hamster hepatocytes: Possible involvement of activator protein-1. *Mol Pharmacol* **50:**556–564
Wadzinski BE, Wheat WH, Jaspers S, Peruski LF, Lickteig RL, Johnson GL and Klemm DJ (1993) Nuclear protein phosphatase 2A dephosphorylates protein kinase A-phosphorylated CREB and regulates CREB transcriptional stimulation. *Mol Cell Biol* **13:**2822–2834
Wera S and Hemmings BA (1995) Serine/threonine protein phosphatases. *Biochem J* **311:**17–29
Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y and Koizumi S (1999) Oxidative stress causes enhanced endothelial cell injury in human heme oxygenase-1 deficiency. *J Clin Invest* **103:**129–135

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