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 isozyme 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 isozymes 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 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 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.
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 Schleicher and Schuell (Dassel, Germany), and Boehringer Mannheim (Mannheim, Germany) and the obtained 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 × 10⁴ cells/well). After 2 h, cells were washed for elimination of nonadherent cells, and cell culture was continued with serum-free medium.

**Bioisotopic 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 [35S]methionine (600 µCi/ml). Cell layers were washed with ice-cold PBS, covered with lysis buffer (PBS, 0.5%; deoxycholic acid, 1%; 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-32P]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 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 [32P]-labeled cDNA was removed after autoradiography by two washing steps with boiling 0.05×/0.1% SSC for 15 min before rebiohybridization.

**cDNA Probes.** The probes were the cDNAs of HO-1, phosphoenolpyruvate carboxykinase (PKC), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of rat (Immenschuh et al., 1998b). The cDNAs were labeled by 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 320 mM sucrose, 3 mM CaCl₂, 2 mM magnesium acetate, 100 µM EDTA, 100 µM PMSF, 150 µM spermide, 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.
tion of 4 ml of buffer A, the nuclei were pelleted by centrifugation at 300g 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 mM KCl, 8.3 mM MgCl₂, 830 mM glycerol, 100 mM EDTA, 100 mM PMSF, 5 mM dithioerythritol, and 10 mM Tris/HCl, pH 8.0 (buffer C).

**Nuclear Run-off Transcription Assay.** The nuclear run-off reaction was performed with 2 × 10⁶ nuclei in a volume of 20 µl as described (Immenschuh et al., 1999b). In the in vitro transcription reaction, the 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% Ficoll, 500 mM EDTA, 100 mM EDTA, 100 mM PMSF, 5 mM dithioerythritol, and 50 mM Tris/HCl, pH 8.0 (buffer C).

**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 (lane 3) or in the presence of OA (10 nM; lane 1), and (C) Kupffer cells (lane 2) or in the presence of OA at concentrations (lane 1) were cultured for 6 h under control conditions (lane 2) or in the presence of OA at concentrations (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 (lane 4). Total cellular 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 Southern blot analysis and sequenced in both directions. 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 32P-labeled cDNAs of HO-1 and GPDH 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.

**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 [35S]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 hepatocytes.
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), CoCl₂ (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 32P-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.).]
Comparative effects of treatment with OA, calyculin A, heme, CoCl$_2$, and Bt$_2$cAMP on HO-1 mRNA expression in primary cultures of rat hepatocytes 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 (Psheenichkin 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$_2$cAMP-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$_2$cAMP 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$_2$cAMP-treated hepatocytes with OA reduced the PCK mRNA expression and enzyme activity elicited by Bt$_2$cAMP dose dependently (Fig. 4). These findings on the PCK gene expression are in agreement with those from a previous report in H4IIE rat hepatocyte cultures (Obrien 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.

**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 increased in a time- and dose-dependent manner by OA in primary cultures of rat hepatocytes.

**TABLE 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fold induction of HO-1 mRNA</th>
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<tbody>
<tr>
<td></td>
<td>6 h</td>
</tr>
<tr>
<td>Control</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>OA (10 nM)</td>
<td>13 ± 1.5*</td>
</tr>
<tr>
<td>Calyculin A (1 nM)</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>Calyculin A (20 nM)</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Heme (10 µM)</td>
<td>40 ± 5*</td>
</tr>
<tr>
<td>CoCl$_2$ (100 µM)</td>
<td>45 ± 6*</td>
</tr>
<tr>
<td>Bt$_2$cAMP (250 µM)</td>
<td>19 ± 2*</td>
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Fig. 3. Inhibition of the OA-dependent HO-1 mRNA induction by KT5720 and synergistic induction of HO-1 mRNA by OA and Bt$_2$cAMP 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$_2$cAMP (100 µM), and a combination of OA plus Bt$_2$cAMP for 6 h. Total RNA was assessed by Northern blot analysis and was sequentially probed with the 32P-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 Bt2cAMP 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 Bt2cAMP-dependent induction of this reporter construct was inhibited by OA (Table 2, pPCK-2500 CAT).

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 Bt2cAMP 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-
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.

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.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Fold Stimulation</th>
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<tr>
<td>1</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>0.1 ± 0.1</td>
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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 Bt2cAMP 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 Bt2cAMP (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 (Roessler et al., 1996). Other
Tf's 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 (Rosenberger et al., 1999). As to the role of ERK 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 LCM 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 HepLa 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 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.

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References


TABLE 2

Regulation of DNA sequences of the rat HO-1 and PCK gene promoter 5'-flanking regions by OA and Bt2cAMP in transiently transfected rat hepatocyte cultures

<table>
<thead>
<tr>
<th>Construct</th>
<th>Fold induction of reporter gene activity</th>
<th>OA</th>
<th>Bt2cAMP</th>
<th>OA + Bt2cAMP</th>
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<tr>
<td>pHO-1338 Luc</td>
<td>2.5 ± 0.2</td>
<td>2.5 ± 0.3</td>
<td>4.5 ± 0.5*</td>
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<tr>
<td>pHO-754 Luc</td>
<td>3 ± 0.5</td>
<td>3.5 ± 1</td>
<td>6 ± 0.4*</td>
<td></td>
</tr>
<tr>
<td>pPCK-5500 CAT</td>
<td>1 ± 0.2</td>
<td>4 ± 0.4**</td>
<td>2.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>pGL3prom Luc</td>
<td>0.1 ± 0.2</td>
<td>0.1 ± 0.1</td>
<td>0.1 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

* indicates significant difference OA versus OA + Bt2cAMP; P ≤ 0.05.* indicates significant difference OA versus Bt2cAMP; P ≤ 0.5.
Immenschuh S, Tan M and Ramadori G (1999) Nitric oxide mediates the lipopoly-

saccharide-dependent upregulation of the heme oxygenase-1 gene expression in
cultured rat Kupffer cells. J Hepatol 30:61–69


oxygenase in pulmonary epithelial cells results in cell growth arrest and in-
creased 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 2:2555–2568

forms of rat liver microsomal heme oxygenase. Only one molecular species of the
enzyme is inducible. J Biol Chem 261:411–419

Masuya Y, Hinko K, Tokunaga R and Taketani S (1998) Involvement of the tyrosine
phosphorylation pathway in induction of human heme oxygenase-1 by hemic,

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 13:17881–17891

and okadaic acid on phosphoenolpyruvate carboxykinase gene expression. Bio-
chem J 303:737–742

phosphatase 1 and 2A differentially regulate the expression of inducible nitric-

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-de-

deficient cells. Proc Natl Acad Sci USA 94:10925–10930

Pakninekpin SP and Wise BC (1995) Okadaic acid increases nerve growth factor
secretion, mRNA stability, and gene transcription in primary cultures of cortical

Roesler WD, Crosson SM, Vinson C and McFie PJ (1996) The α-isform of the
CCAAT/enhancer-binding protein is required for mediating cAMP responsiveness
of the phosphoenolpyruvate carboxykinase promoter in hepatoma cells. J Biol
Chem 271:18688–18694

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 FoxpB 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
Guzehan PS (1988) Regulation of gene expression in adult rat hepatocytes cul-

Shibahara S, Muller R and Taguchi H (1987) Transcriptional control of rat heme

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-methylchol-
anthrene-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 IL, Peruski LF, Lickteig RL, Johnson GL and
Klemm DJ (1993) Nuclear protein-phosphatase 2A dephosphorylates protein ki-

nase A-phosphorylated CREB and regulates CREB transcriptional stimulation.
Mol Cell Biol 13:2822–2834

J 311:17–29

Yachie A, Niida Y, Wada T, Igarashi N, Kaneda H, Toma T, Ohta K, Kasahara Y and
Kisumi S (1999) Oxidative stress causes enhanced endothelial cell injury in

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