Transcriptional activation of the haem oxygenase-1 gene by cGMP via a cAMP response element/activator protein-1 element in primary cultures of rat hepatocytes

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The expression of the rate-limiting enzyme of haem degradation, haem oxygenase-1 (HO-1), can be induced by various stimuli, including lipopolysaccharide, tumour necrosis factor α and NO. The NO signal can be transmitted by cGMP, therefore this study was aimed at testing the activation of the HO-1 gene by cGMP. In primary cultures of rat hepatocytes, both HO-1 mRNA and protein were induced by the NO donor sodium nitroprusside and 8-bromo-cGMP. The HO-1 mRNA induction by cGMP was prevented by the specific protein kinase G inhibitor KT5823. The cGMP-dependent HO-1 mRNA induction was dose-dependent and transcriptionally regulated, as determined by studies with actinomycin D and a nuclear run-on assay. Cycloheximide lowered the cGMP-dependent induction of HO-1 mRNA to about one half. Luciferase reporter constructs driven by about 800 bp of the 5′-flanking region of the rat HO-1 gene were transiently transfected into primary rat hepatocytes; 8-bromo-cGMP caused a 6-fold induction, which was obliterated by deletion and mutation of the cAMP response element/activator protein-1 (CRE/AP-1) ($-665/-654$) site. Thus HO-1 induction by cGMP appears to be stimulated by the protein kinase G pathway and may be mediated mainly via a CRE/AP-1 element in the rat HO-1 promoter.

INTRODUCTION

The microsomal enzyme haem oxygenase (HO) catalyses the first and rate-limiting step of haem degradation producing CO, Fe^{2+} and biliverdin, which is converted into bilirubin by biliverdin reductase [1]. So far, at least two isoenzymes are known: HO-2, which is expressed constitutively, and HO-1, which is inducible and identical with heat-shock protein 32 (HSP32) [2]. HO-1 is induced in response to its substrate haem [2], by various stress stimuli such as UV light, heat [3], sulphydryl-reactive reagents [4], heavy metals, anoxia [5] and endotoxin [6], and by inflammatory cytokines such as tumour necrosis factor α (TNFα), interleukin-1β [7] or interferon-γ [8,9]. Endotoxin and inflammatory cytokines do not induce HO-1 directly, but indirectly via the formation of NO, because their action can be inhibited by inhibitors of NO synthase such as *N*-monomethyl-L-arginine (NMA) [10]. Recently, it was shown that HO-1 activity and mRNA expression could be induced by NO donors such as sodium nitroprusside (SNP) [11], *S*-nitroso-*N*-acetylpenicillamine [12] or 3-morpholinosydnonimine [13] in aortic endothelial cells [12], vascular smooth muscle cells [14] and hepatocytes [15]. Normally, cGMP is the secondary messenger of at least the short-term actions of NO, which activates the haem protein guanylate cyclase [16–20]. Thus it was feasible that cGMP might also mediate the long-term gene-inducing actions of NO. However, in the previous studies on the induction of HO-1 by NO donors, 8-bromo-cGMP did not induce the enzyme either in endothelial cells and smooth muscle cells or in hepatocytes [12,14,15]. This was surprising, because cGMP has been found to participate in the regulation of gene expression; it increased the expression of c-*fos* in BHK cells [21] and TNFα in rat peritoneal macrophages [22,23], and decreased the expression of both transforming growth factor β and fibronectin in mesangial cells [24] and smooth muscle α-actin expression in rat hepatic stellate cells [25].

Thus it was the goal of the present study to reinvestigate whether cGMP, which is the mediator of NO, can activate HO-1 gene expression. Using primary hepatocyte cultures it was found that both the NO donor SNP and the cGMP analogue 8-bromo-cGMP induced HO-1 mRNA expression, reaching a maximum after 6 h. Nuclear run-on experiments indicated that the cGMP-dependent induction of HO-1 was regulated at the transcriptional level. This was corroborated further by identifying a potential cAMP response element/activator protein-1 (CRE/AP-1) site ($-665/-654$) responsive to cGMP in the 5′-flanking region of the HO-1 gene in transient transfection experiments with luciferase gene constructs.

EXPERIMENTAL

Materials

All biochemicals and enzymes were of analytical grade and were purchased from commercial suppliers (details given below).

Abbreviations used: HO-1, haem oxygenase-1; CRE/AP-1, cAMP response element/activator protein-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNFα, tumour necrosis factor α; NMA, *N*-monomethyl-L-arginine; SNP, sodium nitroprusside; FL, *Firefly* luciferase; RL, *Renilla* luciferase; SV40, simian virus 40.

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Cell culture

Cells from male Wistar rats (200–250 g) were used for culture experiments. Hepatocytes were isolated by circulating liver perfusion with collagenase [26]. About 1×10^6 cells were cultured at 37 °C on 6-cm Falcon culture dishes under 95 % air/5 % CO_2 in medium 199 with Earle's salts containing BSA (2 g/l), $NaHCO_3$ (20 mM), Hepes (10 mM), streptomycin sulphate (117 mg/l), penicillin (60 mg/l), insulin (1 nM) and dexamethasone (10 nM). 5 % (v/v) fetal-calf serum was present for the initial 4 h. Cell cultures were incubated in serum-free medium for another 18 h before the experiment. For the experiments, serum-free medium was added containing either SNP or 8-bromo-cGMP for the lengths of time indicated.

RNA isolation, Northern blot analysis and hybridization

Isolation of total RNA and Northern blot analysis was performed as described previously [26]. Autoradiographs of filters were quantified with a PhosphorImager. The cDNAs of rat HO-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or a 28 S RNA oligonucleotide were used as probes for hybridization [26]. cDNAs were labelled with [α-^{32}P]dCTP and the oligonucleotide was labelled with [γ-^{32}P]dATP using commercial labelling kits according to the manufacturer's instructions.

Isolation of nuclei from rat hepatocyte cultures and nuclear run-on transcription assay

Hepatocytes (1×10^7) were used for isolation of nuclei for the nuclear run-on assay. The isolation procedure was performed as described previously [27]. The nuclear run-on reaction was performed with 2×10^6 nuclei in a vol. of 20 μl. The *in vitro* transcription reaction was started by the addition of 30 μl of 58 % (v/v) glycerol, 150 mM NH_4Cl, 8.3 mM $MgCl_2$, 830 μM $MnCl_2$, 70 μM EDTA, 25 units RNAsin, 830 μM ATP, 830 μM CTP, 830 μM GTP, 100 μCi of [^{32}P]UTP and 33 mM Hepes, pH 8.0. After incubation of nuclei for 30 min at 37 °C, the reaction was stopped by the addition of 10 μl of EDTA (100 mM). RNA extraction, prehybridization and hybridization were performed as described previously [28].

Plasmid constructs

All basic recombination techniques referred to are standard protocols [29]. The rat HO-1 promoter 5′-flanking region [30] from −754 to +71 was amplified by PCR from rat genomic DNA by using the oligonucleotide 5′-GCCAGGAATTC-GGAGGGTAATTGTCCAAG-3′ (−754/−726) as forward and 5′-GAGATGGCTCTGCTCCGGCAGGCTCCACTC-3′ (+42/+71) as reverse primer respectively. The resulting PCR product was rendered blunt-ended by Klenow enzyme, phosphorylated with T4 polynucleotide kinase and ligated into the *Sma*I site of pUC18. The insert was then excised with *Kpn*I/*Bam*HI and cloned into the *Kpn*I/*Bgl*II site of pGl3basic (Promega) (pHO-754; construct 1, see Figure 5). In construct pHO-754del (construct 2; see Figure 5), the −714/−549 fragment was deleted by partial digestion with *Hin*dIII. The construct pHOΔCRE/AP-1 was constructed by using PCR-based mutagenesis. The forward primer 5′-TAGACTCCGGTACTCAG-GCA-3′ (−658/−639), containing a C to T and a T to A conversion at positions 1 and 4 respectively, and the reverse primer 5′-CACATGGCTCTGACACATCTATAAC-3′, containing the wild-type HO-1 sequence −668/−692, were used with the ExSite Mutagenesis kit (Stratagene) to generate pHOΔCRE/AP-1 (construct 3; see Figure 5). All constructs were verified by sequencing in both directions.

Cell transfection and luciferase assay

Rat hepatocyte cultures (about 1×10^6 cells per dish) were transiently transfected with 2.5 μg of plasmid DNA containing 500 ng of pRL-simian virus 40 (SV40) (Promega) to control transfection efficiency and 2 μg of the appropriate HO-1 promoter *Firefly* luciferase (FL) construct. The pRL-SV40 contains the *Renilla* luciferase (RL) gene under control of the SV40 promoter, which allows the constitutive expression of the RL gene. In every culture experiment, two dishes were transfected per measured point. The DNA was transfected as a calcium phosphate precipitate [28] in the presence of 4 % (v/v) newborn-calf serum/125 mM $CaCl_2$/2 × Hepes (pH 7.05) for 5 h. After removal of the media, cells were cultured under standard conditions without serum. After 24 h, cells were treated with fresh media containing 8-bromo-cGMP and were cultured for another 12 h. Cells were then washed twice with 0.9 % (w/v) NaCl and incubated for 15 min on a rocking platform with 300 μl of passive lysis buffer supplied with the dual luciferase assay kit (Promega). The lysate was then scraped from the plates, vortex-mixed for 20 s and centrifuged for 2 min. From the supernatant, 20 μl was then assayed for FL activity in a luminometer (Berthold, Pforzheim, Germany) by injection of 100 μl of Luciferase Assay Reagent II supplied with the dual luciferase assay kit (Promega). After addition of 100 μl of Stop and Glo™ Reagent (Promega), which quenches the activity of FL, the RL activity was recorded again in a luminometer (Berthold).

Western blot analysis

After washing the cell cultures twice with 0.9 % (w/v) NaCl, total protein was prepared from 1×10^6 hepatocytes by the addition of 1 ml of boiling lysis buffer [0.1 % (w/v) SDS/10 mM Tris (pH 7.4)] and subsequent scraping of the cells. Cells were then 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 by the method of Bradford. Total protein (40 μg) was loaded on to an SDS/polyacrylamide (10 %) gel and blotted on to nitrocellulose membranes after electrophoresis. Membranes were blocked with Tris-buffered saline [30 mM Tris/HCl (pH 7.5)/150 mM NaCl] containing 1 % (w/v) BSA, 10 mM Tris/HCl, pH 7.5, and 0.1 % (v/v) Tween for 1 h at room temperature. The primary antibody against rat HO-1 (Stressgene, Vancouver, BC, Canada) was then added in a 1:1000 (by vol.) dilution and the blot was incubated for 12 h at 4 °C. The ECL Western blotting system (Amersham) was then used for detection.

RESULTS

Induction of HO-1 mRNA expression by SNP and 8-bromo-cGMP in a time- and dose-dependent manner in rat hepatocyte cultures

Primary rat hepatocyte cultures were treated with the NO-releasing agent SNP and the cGMP analogue 8-bromo-cGMP. In hepatocytes cultured for up to 24 h with SNP and 8-bromo-cGMP, HO-1 mRNA was induced by both agents with a peak occurring at 6 h (Figure 1A). Both SNP and 8-bromo-cGMP induced the HO-1 mRNA expression in a dose-dependent manner, reaching maximal action at 10 μM and 200 μM respectively (Figure 1B). When SNP was applied at concentrations higher than 100 μM, cell viability was significantly lowered, as judged by light microscopy and lactate dehydrogenase leakage (results not shown). HO-1 mRNA was up-regulated 14-fold by 10 μM SNP and 7-fold by 200 μM 8-bromo-cGMP within 6 h (Figures 1A and 1C). Simultaneous treatment with 8-bromo-

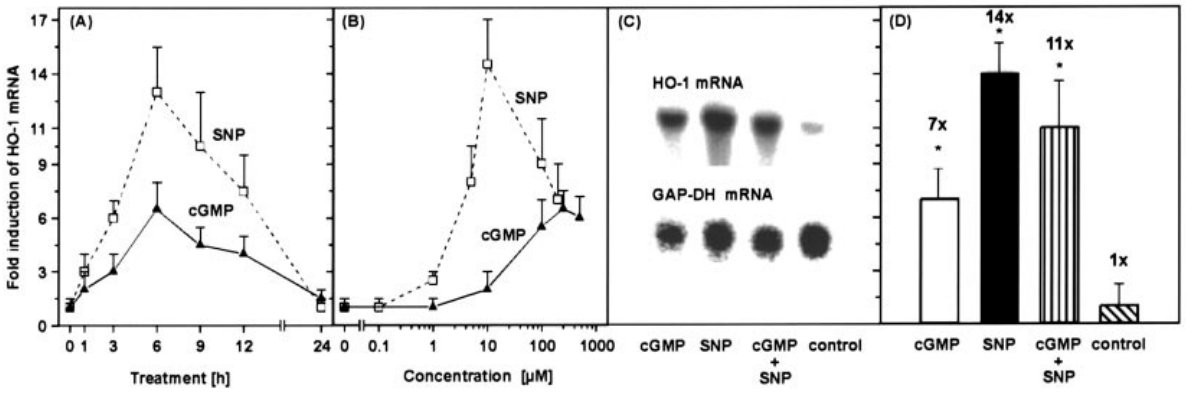

Figure 1 Induction of HO-1 mRNA expression in primary rat hepatocyte cultures by SNP and 8-bromo-cGMP

Rat hepatocyte cultures were treated for 18 h with serum-free medium. Then the culture was continued (**A**) in the presence of SNP (10 μM) and 8-bromo-cGMP (100 μM) for the times indicated, or (**B**) for 6 h in the presence of increasing concentrations of SNP (0.1–200 μM) or 8-bromo-cGMP (1–500 μM). Total cellular RNA (15 μg) was subjected to slot-blot analysis and sequentially probed with the ^{32}P-labelled cDNA of HO-1 and glyceraldehyde-3-phosphate dehydrogenase. Autoradiograms were quantified as described in the Experimental section. Data in (**A**) and (**B**) are from at least three independent experiments; numbers show the fold-induction rate relative to the basal HO-1 mRNA expression at 0 h $\pm$ S.E.M. (**C**) Hepatocytes were cultured for 18 h in serum-free medium, and then as indicated in the presence of 100 μM 8-bromo-cGMP, 10 μM SNP or 8-bromo-cGMP plus SNP for a further 6 h. Total cellular RNA (15 μg) was analysed by Northern blotting and quantified as in (**A**) and (**B**). (**D**) Values $\pm$ S.E.M. represent the fold induction of HO-1 mRNA normalized to GAPDH mRNA levels of three independent experiments. The data were fitted to the Student's *t* test for paired values: *, significant difference of control versus cGMP, SNP or SNP + cGMP; $P \leqslant 0.05$.

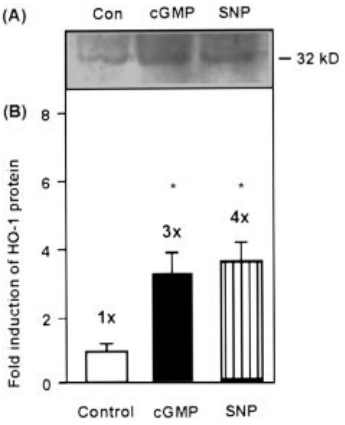

Figure 2 Induction of HO-1 protein in primary rat hepatocyte cultures by SNP and 8-bromo-cGMP

Rat hepatocyte cultures were for 18 h in serum-free medium. Then, cultures were left untreated (con) or were treated for 12 h with 200 μM 8-bromo-cGMP (cGMP) or 10 μM SNP. (**A**) A representative Western blot; 40 μg of total protein from rat hepatocyte cultures was subjected to Western blot analysis with an antibody for rat HO-1 protein, as described in the Experimental section. (**B**) Blots were quantified with a videodensitometer. Values $\pm$ S.E.M. represent the fold induction of HO-1 protein of three independent experiments. Statistics used, Student's *t* test for paired values: *, significant difference for control versus cGMP or SNP; $P \leqslant 0.05$.

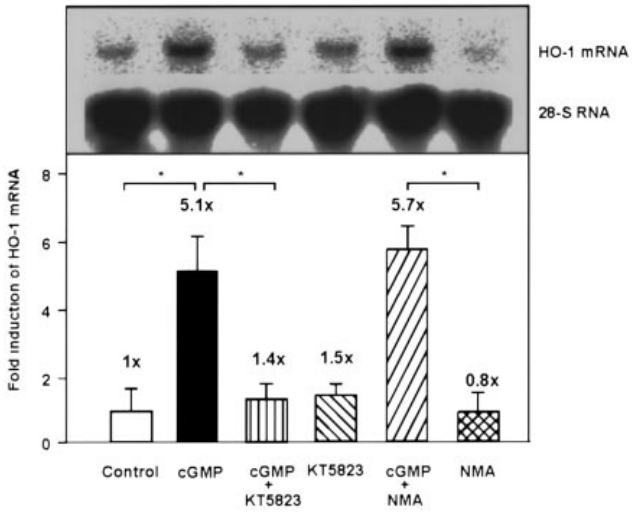

Figure 3 Inhibition of the 8-bromo-cGMP-dependent HO-1 mRNA induction by the protein kinase G inhibitor KT5823, but not the NO synthase inhibitor NMA in primary rat hepatocyte cultures

Primary rat hepatocytes were cultured for 6 h with 200 μM 8-bromo-cGMP, 1 μM KT5823, 250 μM NMA or with a combination of 200 μM 8-bromo-cGMP + 1 μM KT5823 or 200 μM 8-bromo-cGMP + 250 μM NMA. (**A**) Representative Northern blot; total cellular RNA (15 μg) was subjected to Northern blot analysis and was probed with the ^{32}P-labelled cDNA of HO-1 and subsequently with a 28 S RNA oligonucleotide. (**B**) Autoradiograms were quantified with a phosphorimager and the signal of the 28 S RNA band served as an internal standard. Values $\pm$ S.E.M. represent the fold induction of HO-1 mRNA normalized to 28 S RNA levels of three independent experiments. Statistics, Student's *t* test for paired values: *, significant differences of control versus cGMP, cGMP versus cGMP + KT5823 or NMA versus cGMP + NMA, $P \leqslant 0.05$.

cGMP plus SNP had no additive effect on the magnitude of HO-1 mRNA induction (Figure 1D). The increase of HO-1 mRNA expression induced by both cGMP and SNP was also observed on the protein level, as determined by Western blot analysis (Figure 2).

In further experiments, it was investigated whether cGMP induced HO-1 mRNA via activation of protein kinase G. When hepatocyte cultures were treated with the protein kinase G inhibitor KT5823 [30], the cGMP-dependent induction of HO-1 mRNA was significantly lowered (Figure 3 and Table 1). No inhibition by KT5823 was observed for the induction of HO-1 mRNA by haem and cadmium chloride (Table 1). Treatment with the NO synthase inhibitor NMA did not affect the cGMP-dependent induction of HO-1 mRNA (Figure 3); thus it appeared

Table 1 Comparative effects of treatment with KT5823 on 8-bromo-cGMP-, haem- and $CdCl_2$-dependent HO-1 mRNA induction 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 h. Total RNA was isolated as decribed in the Experimental section and subjected to Northern blot analysis. Numbers 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 ± S.E.M.. Student's *t* test for paired values: *, significant differences control versus 8-bromo-cGMP, control versus $CdCl_2$, control versus haem, control versus KT5823 + haem and control versus KT5823 + $CdCl_2$, $P \leqslant 0.05$.

Treatment	Fold induction of HO-1 mRNA
Control	1 ± 0.2
8-Bromo-cGMP (200 μM)	6 ± 0.3*
$CdCl_2$ (10 μM)	32 ± 2*
Haem (10 μM)	30 ± 3*
KT5823 (1 μM)	1.5 ± 0.1
KT5823 (1 μM) + 8-bromo-cGMP (200 μM)	1.4 ± 0.2
KT5823 (1 μM) + haem (10 μM)	28 ± 2*
KT5823 (1 μM) + $CdCl_2$ (10 μM)	33 ± 3*

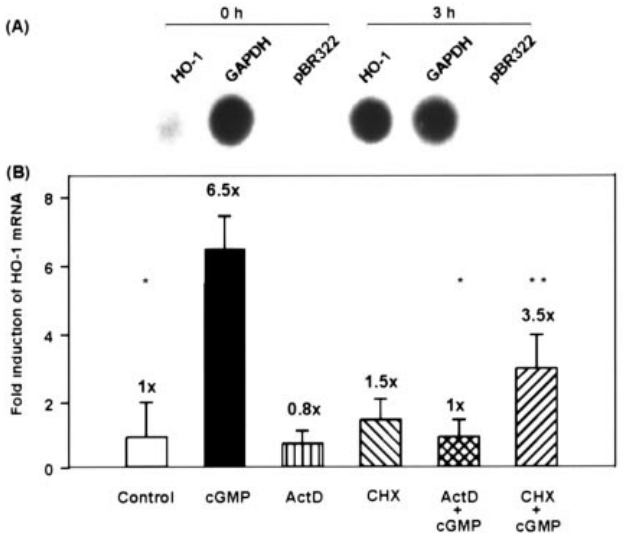

Figure 4 Induction of HO-1 transcription by 8-bromo-cGMP and inhibition of the 8-bromo-cGMP-dependent HO-1 mRNA induction by actinomycin D and cycloheximide

(**A**) A representative nuclear run-on assay demonstrating the induction of HO-1 gene transcription by 8-bromo-cGMP. Nuclei from hepatocyte cultures either non-treated or treated for 3 h with 8-bromo-cGMP (200 μM) were subjected to a nuclear run-on transcription assay. [^{32}P]UTP-labelled nascent RNA transcripts were purified and hybridized to HO-1 cDNA, GAPDH cDNA or the plasmid pBR322 as a control. (**B**) Hepatocytes were pretreated for 30 min with actinomycin D (ActD; 1 μg/ml) or cycloheximide (CHX; 1 μg/ ml) and after adding 8-bromo-cGMP, cell culture was continued for 6 h. Total RNA (15 μg) was subjected to Northern analysis, as in Figure 1. Values ± S.E.M. represent the fold induction of HO-1 mRNA normalized to GAPDH levels of three independent experiments. Statistics, Student's *t* test for paired values: *, significant differences of control versus cGMP, ActD + cGMP versus cGMP; **, CHX + cGMP versus cGMP, $P \leqslant 0.05$.

that cGMP acted directly via the activation of protein kinase G, and not via production of NO.

Induction by 8-bromo-cGMP of HO-1 gene transcription in rat hepatocyte cultures

The transcriptional rate of HO-1 gene expression in primary rat hepatocytes after treatment with 8-bromo-cGMP was determined by a nuclear run-on assay. The HO-1 gene transcriptional rate was induced approx. 6-fold by 8-bromo-cGMP after 3 h, while that of the GAPDH gene (studied as a control) was not altered (Figure 4A). The transcriptional mechanism of HO-1 gene activation by 8-bromo-cGMP was verified by pretreatment of cell cultures with the transcriptional inhibitor actinomycin D; it prevented the 8-bromo-cGMP-dependent HO-1 mRNA induction (Figure 4B). The protein synthesis inhibitor cycloheximide lowered the 8-bromo-cGMP-dependent up-regulation of HO-1 mRNA by about 50% (Figure 4B), suggesting that protein synthesis *de novo* is, in part, also involved in the induction of the HO-1 mRNA. These data indicate that the 8-bromo-cGMP-dependent induction of HO-1 mRNA was regulated mainly at the transcriptional level.

Regulation of transfected rat HO-1 promoter luciferase gene constructs by 8-bromo-cGMP in rat hepatocyte cultures

To corroborate the conclusion that HO-1 gene expression was regulated by cGMP at the transcriptional level, luciferase gene constructs driven by the DNA sequence from −754 to +71 of the rat HO-1 gene 5′-flanking region [31] (Figure 5, construct 1; pHO-754) containing a prostaglandin J_2 element (−673/−668), a heat-shock element (−278/−263) and a putative CRE/AP-1 site (−665/−654) were transfected transiently into primary rat hepatocytes. The transfected hepatocyte cultures exhibited a 10-fold higher luciferase activity in comparison with the control vector carrying the luciferase gene driven by the minimal SV40 promoter (pGL3prom) (Figure 5, construct 1 versus construct 4). Luciferase expression of pHO-754 was up-regulated by about 6-fold by 8-bromo-cGMP (Figure 5, construct 1). In contrast, no induction of luciferase activity was observed with the SV40 promoter control construct after treatment with 8-bromo-cGMP (Figure 5, construct 4). Because the activation by cGMP of the human c-*fos* promoter was shown to be regulated either by a serum response element, a CRE or an AP-1 element [21], a mutant luciferase construct with a *Hin*dIII-catalysed deletion from −714 to −549 of the 5′-flanking region, i.e. without the CRE/AP-1 site and the prostaglandin J_2 element (Figure 3, construct 2; pHO-754del) and a construct lacking the CRE/AP-1 site (Figure 5, construct 3; pHOΔCRE/AP-1) was transfected into hepatocytes. With both mutated gene constructs, the 8-bromo-cGMP-dependent up-regulation of luciferase activity was abolished (Figure 3). These results suggest that the CRE/AP-1 (−665/−654) site (5′-CTGACTTCAGTC-3′) in the 5′-flanking region of the HO-1 gene conferred the responsiveness of the HO-1 gene constructs to 8-bromo-cGMP.

DISCUSSION

The present investigation has shown that in primary cultured hepatocytes the second messenger cGMP induced HO-1 gene transcription with a similar time course as the NO-releasing agent SNP. The cGMP-dependent HO-1 mRNA induction appeared to occur via activation of the protein kinase G pathway, and was mainly transcriptionally regulated. In transfection studies with luciferase gene constructs driven by the 5′-flanking region of the rat HO-1 gene promoter, a CRE/AP-1 (−665/−654) site could be identified which mediated the cGMP response.

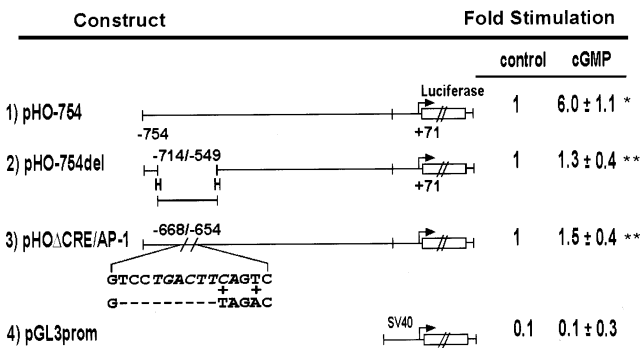

Figure 5 Analysis of rat HO-1 gene sequences involved in the induction by 8-bromo-cGMP in transiently transfected rat hepatocyte cultures

The indicated rat HO-1 gene sequences from the 5′-flanking part were cloned in front of the luciferase reporter gene. These constructs were transiently transfected into primary rat hepatocyte cultures. After 24 h, the transfected cells were treated for 12 h with 200 μM 8-bromo-cGMP. In each experiment, the fold stimulation of luciferase activity by cGMP was determined relative to the control, which was set equal to 1. The values represent means ± S.E.M. of three independent experiments. Student's *t* test for paired values: *, significant differences control versus cGMP; **, construct 1 versus construct 2 and construct 1 versus construct 3, $P \leqslant 0.05$. In construct 3, the sequence representing the CRE/AP-1 site is indicated in italics, mutated bases are indicated by + and deleted bases are shown as a dashed line. H, *Hin*dIII restriction site

HO-1 mRNA induction by cGMP in rat hepatocyte cultures

Induction of HO-1 gene expression by cGMP has not been described so far. Furthermore, 100 μM cGMP (the effective dose used in this study) induced neither HO-1 activity in endothelial cells [12] nor HO-1 mRNA expression in vascular smooth muscle cells [14,32]. In hepatocytes, a cGMP concentration of 20 μM, which was in the lower range of effective concentrations used in this study (Figure 1), was found to be ineffective in the induction of HO-1 gene expression [15]. The apparent inability of cGMP to induce HO-1 in both endothelial and smooth muscle cells, in contrast to hepatocytes, might indicate that the induction by cGMP could be dependent on the cell type. This is supported further by the finding that treatment with cGMP did not affect HO-1 gene expression in the hepatoma cell line H-35 or in NIH-3T3 fibroblasts (results not shown). The inhibition of the cGMP-dependent induction of HO-1 mRNA by the protein kinase G inhibitor KT5823 was specific, because both the haem- and cadmium-dependent HO-1 mRNA induction was not influenced (Figure 3 and Table 1). This suggests that haem and cadmium chloride may mediate their effects on HO-1 gene activation via a different signalling pathway from that of cGMP.

cGMP in the regulation of gene expression via transcription factors

A number of response elements in the mouse and human HO-1 promoter and 5′-flanking region have been identified (for review, see [9]), whereas the rat HO-1 promoter has not been characterized in detail yet. The response elements mediating cGMP-dependent transcriptional activation, in general, are poorly defined. In transfection experiments with constructs containing either multiple serum response elements (5′-CCATATTAGG-3′), AP-1-binding sites (5′-TGACTCA-3′) or CREs (5′-TGACGTCA-3′) from the human c-*fos* promoter in front of a thymidine kinase promoter and the chloramphenicol acetyltransferase reporter gene, it was found that cGMP/protein kinase G stimulated the expression of all reporter gene constructs to the same extent [21]. The sequence 5′-TGACTTCAGT-3′ identified in this study contains the CRE consensus sequence (5′-TGACGTCA-3′) in 7 out of 8 bp and, additionally, the AP-1-binding site (5′-TGACTCA-3′; 6 out of 7 bp matched). Therefore the sequence that mediated the regulation by cGMP of the rat HO-1 promoter was named CRE/AP-1 element. The fact that the cGMP-dependent HO-1 induction was inhibited by cycloheximide indicates that this mechanism depends on protein synthesis *de novo*. Thus protein products of immediate early genes, such as c-Jun/c-Fos, may be involved in this process.

Physiological role of the cGMP-dependent induction of HO-1

The cGMP-dependent induction of HO-1 is of physiological significance for a number of reasons. Because HO-1 enzymically degrades the pro-oxidant haem and produces the anti-oxidant bilirubin [33], the induction of HO-1 expression is considered a protective response against haem-mediated oxidative stress. The induction of HO-1 *in vivo* provided protection against glycerol-induced rhabdomyolysis [34]. A protective effect of HO-1 against cytotoxicity has been demonstrated directly in coronary endothelial cell cultures in which the haem- and haemoglobin-dependent toxicity was efficiently attenuated by overexpression of the HO-1 gene [35]. In addition, it was shown that up-regulation of the HO-1 gene protected against non-haem-mediated oxidative stress, such as that elicited by UV light [3]. Intriguingly, it was demonstrated in an *in vivo* model of experimental pleurisy that the induction of HO-1 and the pharmacological modification of HO-1 enzyme activity may be significant for the endogenous cellular protection against inflammation [36]. Thus stimulation of the HO-1 gene expression by physiological stimuli, such as cGMP, may provide an additional means of protection against oxidative injuries.

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