Kidney International, Vol. 51 (1997), pp. 564–566

The hypoxia-inducible factor-1 DNA recognition site is cAMP-responsive

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The hypoxia-inducible factor-1 DNA recognition site is cAMP-responsive. The hypoxia-inducible factor-1 (HIF-1) was first described as a DNA binding activity that specifically recognizes an 8 bp hypoxia response element (HRE) known to be essential for oxygen-regulated erythropoietin gene expression. In electrophoretic mobility shift assays (EMSAs) HIF-1 DNA binding activity is only detectable in nuclear extracts of cells cultivated in a low oxygen atmosphere. In addition to HIF-1, a constitutive DNA binding activity also specifically binds the HIF-1 probe. Based on EMSAs using competitor oligonucleotides, specific antibodies and recombinant proteins, we previously reported that the constitutive HRE binding factor is composed of ATF-1 and CREB-1. Here we show that this site is functionally responsive to the cAMP agonist 8Br-cAMP in a dose-dependent manner under hypoxic but not under normoxic conditions. These results were confirmed by using the protein kinase A (PKA) activator *Sp*-cAMPS and the PKA inhibitor *Rp*-cAMPS: while *Sp*-cAMPS was synergistic with hypoxia on the HIF-1 DNA recognition site, the *Rp*-cAMPS isomer showed no effect. Our findings suggest that the PKA-signaling pathway is enhancing oxygen-dependent gene expression via the HRE.

Oxygen-regulated expression of the erythropoietin (Epo) gene in fetal liver and adult kidney is mediated by a hypoxia response element (HRE) located in the 3′ flanking region of the Epo gene [1]. Using site-directed mutagenesis, an 8 bp motif has been defined that is crucial for effector function of the HRE. Electrophoretic mobility shift assays (EMSAs) using a labeled HRE probe detected a hypoxia-inducible factor (HIF-1) whose DNA binding activity is strongly induced in extracts from cells exposed to reduced oxygen concentrations [1]. Recent biochemical purification and cloning revealed that HIF-1 is composed of two subunits, α and β, both belonging to the basic-helix-loop-helix-PAS family of transcription factors [2, 3]. While HIF-1β is identical to the aryl hydrocarbon receptor nuclear translocator (ARNT), human and murine HIF-1α represent novel members of this family [3, 4]. Besides this hypoxia-inducible factor, a specific constitutive HRE binding activity is also present in these EMSAs [1–3]. We previously reported that: (i) a cAMP-responsive element (CRE)-containing oligonucleotide efficiently competed for binding of the HIF-1 probe to this constitutive factor; (ii) monoclonal antibodies raised against the CRE binding factors ATF-1 and CREB-1 supershifted the constitutive factor; and (iii) recombinant ATF-1 and CREB-1 proteins bound HIF1 probes either as homodimers or as heterodimers [5]. Since these transcription factors are known to mediate the effects of the cAMP signaling cascade [6–8], we investigated whether the HRE site might confer a cAMP-dependent regulation of gene expression.

Methods

Cell culture, transient transfection and reporter gene assays were performed as described in detail previously [5]. Briefly, HeLa cells were co-transfected with each 25 μg of a luciferase reporter plasmid and a β-galactosidase expression vector (pCMVβgal), which allows a correction for differences in transfection efficiency and extract preparation. Ten minutes after electroporation, the cells were split, incubated for 32 to 37 hours at normoxic (20% O_2) or hypoxic (1% O_2) conditions, and lysed for determination of reporter activity. The pGLEPOHRE.3 vector contained three copies of the EPO HRE downstream of the luciferase gene which was driven by a heterologous SV40 promoter [5]. As controls, the parental vector pGL3Promoter (Promega) and pGLEPOHREmt.3, which contained three mutated copies of the EPO HRE [5], were included. The cAMP derivatives used included 8-bromoadenosine-3′,5′-cyclic monophosphate (8Br-cAMP) and the isomers *Sp*-adenosine-3′,5′-cyclic monophosphothioate (*Sp*-cAMPS) and *Rp*-adenosine-3′,5′-cyclic monophosphothioate (*Rp*-cAMPS).

Results

The cAMP agonist 8Br-cAMP enhances HRE function

To functionally assess the cAMP responsiveness of the EPO HRE, a luciferase reporter gene containing three copies of a HIF-1 DNA binding oligonucleotide (pGLEPOHRE.3) was transiently transfected into HeLa cells. Subsequently, the cells were treated with different concentrations of the cAMP agonist 8Br-cAMP and cultured at normoxic or hypoxic conditions. As shown in Figure 1, hypoxic reporter gene expression was enhanced by 8Br-cAMP in a dose-dependent manner, reaching the highest value at 1 mM. In contrast, normoxic luciferase expression remained almost unaffected within this concentration range, indicating that 8Br-cAMP effects are mediated by the HRE. To directly demonstrate that the HRE is involved in this effect, HeLa cells were also transfected with either the empty vector (pGL3Promoter) alone, or with a reporter plasmid containing three mutant EPO HRE sites (pGLEPOHREmt.3) [5]. As shown in Figure 2, after transfection with pGLEPOHRE.3 hypoxia alone increased luciferase expression by 6.3-fold ($P < 0.01$, $N = 3$, t-test). Luciferase expression in cells transfected with pGLEPOHREmt.3 was not significantly different from the empty

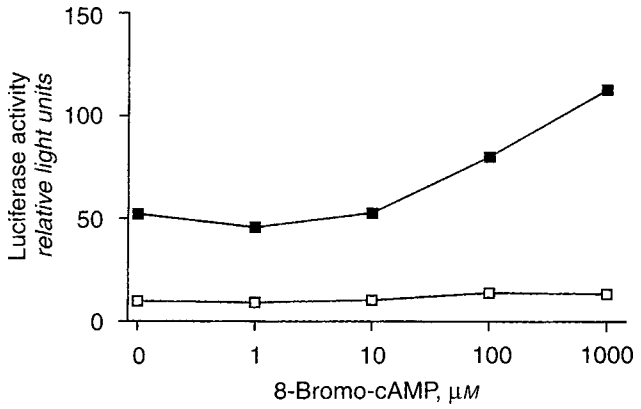

Fig. 1. *Dose-dependent enhancement of hypoxic luciferase induction by 8Br-cAMP.* A luciferase reporter construct (pGLEPOHRE.3) containing 3 HREs derived from the EPO gene 3′ flanking region was transiently transfected into HeLa cells which were split and exposed to either normoxic or hypoxic conditions. Cells were treated with increasing amounts of 8Br-cAMP as indicated. Symbols are: (□) 20% O_2; (■) 1% O_2.

control vector, confirming that hypoxic induction is dependent on the presence of HREs (Fig. 2). A weak hypoxic induction, however, was already observed with the pGL3Promoter plasmid, indicating that the SV40 promoter is slightly induced by hypoxia in this cell line. However, one copy of the HRE oligonucleotide was not sufficient to significantly enhance hypoxic reporter gene expression [5]. After treatment with the cAMP agonist 8Br-cAMP (1 mM), hypoxic luciferase expression was enhanced by 64% ($P < 0.005$, $N = 3$, t-test), whereas pGL3Promoter- and pGLEPOHREmt.3-transfected cells did not significantly induce luciferase expression. Therefore, our results indicate that the HRE is not only hypoxia-responsive but also cAMP-responsive.

A cAMP antagonist does not affect hypoxic reporter gene expression

To examine whether cAMP potentiates hypoxic inducibility as a result of an independent signaling pathway or as part of the hypoxia signaling cascade itself, we repeated the experiments mentioned above using the two isomers *Sp*-cAMPS (a cAMP agonist) and *Rp*-cAMPS (a cAMP antagonist). If cAMP were involved in the oxygen signal transduction pathway, a cAMP antagonist would decrease hypoxic inducibility of pGLEPOHRE.3. In contrast, if cAMP were part of a parallel pathway leading to enhanced expression after exposure to hypoxia, a cAMP antagonist would have no effect on reporter gene expression. As shown in Figure 3, after incubation of transiently transfected HeLa cells with 100 μM of the corresponding cAMP isomers for 32 to 37 hours, the cAMP agonist *Sp*-cAMPS, similar to 8Br-cAMP, enhanced hypoxic reporter gene induction by 50% ($P < 0.05$, $N = 3$, t-test), whereas the cAMP antagonist *Rp*-cAMPS had no significant effect on hypoxic induction. As for 8Br-cAMP, *Sp*-cAMPS neither had an effect under normoxic conditions, nor were the HRE-deficient control plasmids responsive to this agent.

Conclusions

The results presented here confirm our previous observation suggesting that the HRE is cAMP-responsive under hypoxic, but not under normoxic conditions [5]. Our observation that the cAMP antagonist *Rp*-cAMPS does not influence hypoxic inducibility (Fig. 3) indicates that cAMP is not a crucial element of the hypoxia-activated signal transduction pathway *per se*. However, hypoxic induction of gene expression might act in concert with other signaling pathways thereby enhancing the response to low oxygen concentrations. The cAMP signaling cascade was also found to be implicated in hypoxic EPO [9, 10] and lactate

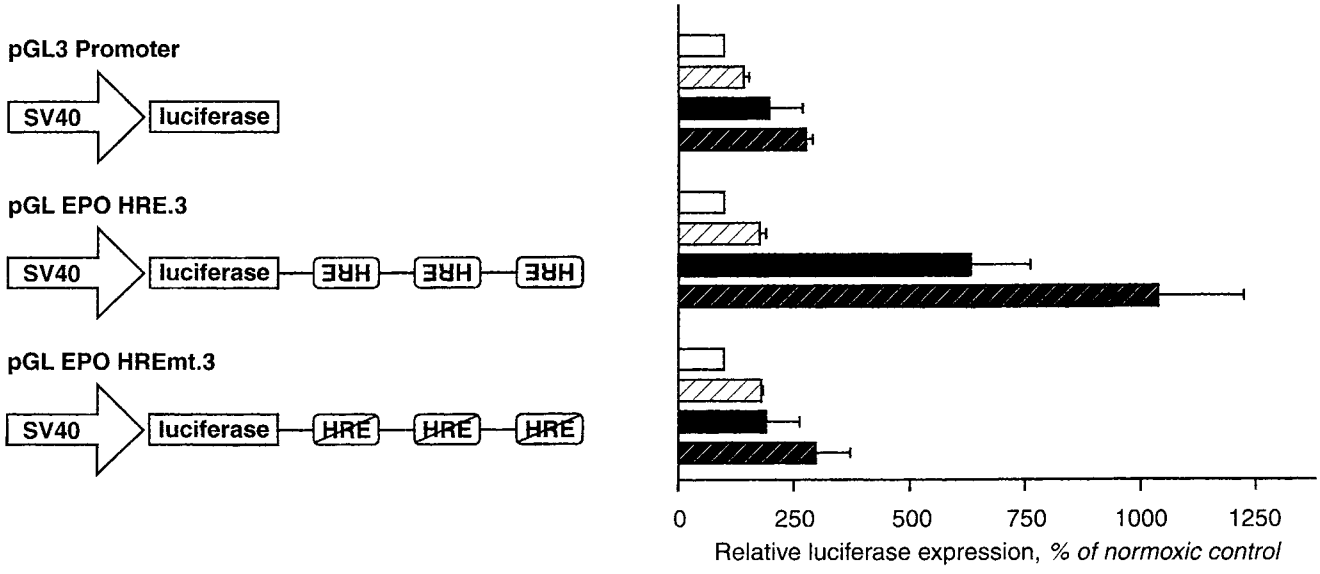

Fig. 2. *8Br-cAMP effects are dependent on functional HREs.* Transiently transfected HeLa cells were treated with 1 mM 8Br-cAMP and cultured at 20% or 1% O_2. Copy number and orientation of the HREs derived from the EPO gene are indicated. Reporter gene expression is shown as percentage of the respective untreated normoxic controls (means ± SD of 3 independent experiments). Normalization to the co-transfected β-galactosidase was inpracticable since the pCMVβgal vector itself was activated by 1 mM (but not by 0.1 mM) 8Br-cAMP (I.K., unpublished observations). Symbols are: (□) 20% O_2; (▨) 20% O_2 + 8Br-cAMP; (■) 1% O_2; ([▨]) 1% O_2 + 8Br-cAMP.

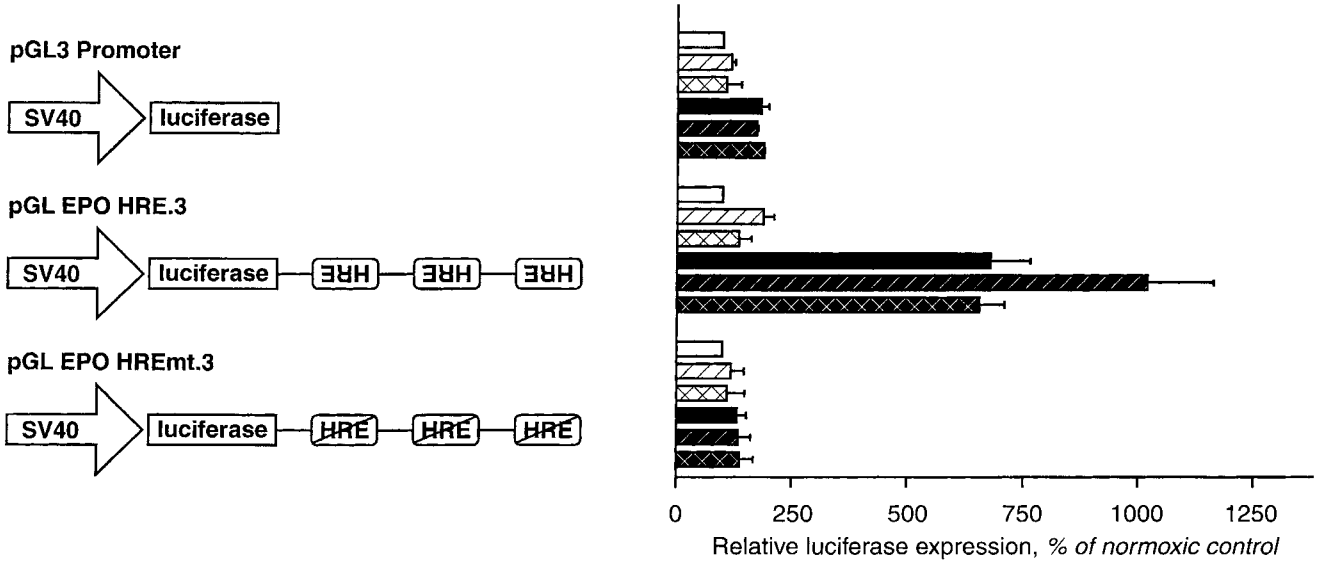

Fig. 3. *Hypoxic induction of reporter gene expression is independent of treatment with a cAMP antagonist.* HeLa cells were transfected and cultured as mentioned in Figure 2. Each 100 μM of a cAMP agonist (*Sp*-cAMPS) or the isomeric antagonist (*Rp*-cAMPS) were added to the cells. Copy number and orientation of the HREs derived from the EPO gene are indicated. Reporter gene expression is normalized to the β-galactosidase activity derived from the co-transfected β-galactosidase expression vector since at the used concentrations of cAMP analogues, no regulation of pCMVβgal could be observed. Results are shown as percentage of the respective untreated normoxic controls (means ± SD of 3 independent experiments). Symbols are: (□) 20% O_2; (▨) 20% O_2 + *Sp*-cAMPS; (⊠) 20% O_2 + *Rp*-cAMPS; (■) 1% O_2; ([▨]) 1% O_2 + *Sp*-cAMPS; (⊠) 1% O_2 + *Rp*-cAMPS.

dehydrogenase (LDH) induction [11]. Recently, Firth, Ebert and Ratcliffe reported the presence of a CRE separated by only 14 bp from a HRE in the LDH promoter [12]. The adenylate cyclase activator forskolin enhanced hypoxic induction of gene expression mediated by the HRE but reduced normoxic expression. Mutation of the CRE site abolished forskolin-dependent synergy with hypoxia, suggesting that the target sites of hypoxia and cAMP signal transduction pathways reside in close vicinity on the LDH promoter [12]. In contrast, our data provide evidence that, at least in the EPO 3′ enhancer, the cAMP effects might also be mediated by the HRE itself.

Acknowledgments

This project was supported by Swiss National Science Foundation Grant 31-36369.92, the Julius Klaus-Stiftung and the Olga Mayenfisch Stiftung. R.H.W. is a recipient of the "Sondermassnahmen des Bundes zur Förderung des akademischen Nachwuchses." We thank S. Kozlov for the gift of pCMVβgal and C. Gasser for the artwork.

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