

Co-Transactivation of the 3' Erythropoietin Hypoxia Inducible Enhancer by the HIF-1 Protein

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ABSTRACT: Erythropoietin (Epo) is a glycoprotein hormone that is the primary regulator of red blood cell production. Epo production increases in response to tissue hypoxia. This increase occurs primarily at the transcriptional level. Hypoxia inducible factor (HIF-1) is a DNA binding protein that binds to a hypoxia inducible enhancer in the 3' flanking sequence of the Epo gene. HIF-1 is a heterodimer that consists of an α and β subunit. HIF-1 DNA binding activity is induced in response to hypoxia. In order to determine if one or both HIF-1 subunits is capable of ligand binding, subsequently leading to Epo production we performed co-transactivation experiments. Transfections were performed in Hep 3B, an Epo producing human hepatoma cell line and Cos-7, a non-Epo producing monkey kidney cell line. Cells were co-transfected with the 38 bp Epo enhancer fragment bearing the HIF-1 binding motif, subcloned in the luciferase reporter plasmid and either the HIF-1 α cDNA, HIF-1 β cDNA, HIF-1 α and HIF-1 β cDNAs or pREP-4 respectively. Cells were incubated in an hypoxic (1% O₂) or normoxic (21% O₂) environment and assayed for luciferase activity. Epo levels were measured in the culture media from the transfected plates by an ELISA assay. Under hypoxic conditions Hep 3B cells transfected with the HIF-1 α cDNA alone showed a 2.2 fold increase in luciferase activity, HIF-1 β showed a 3.4 fold increase and cells transfected with HIF-1 α and β showed a 6.9 fold increase in activity over cells transfected with pREP-4. The baseline luciferase activity in transfected 3B cells incubated in normoxia was very low. However, a similar fold increase in luciferase activity in cells transfected with both HIF-1 α and β was noted. Under normoxic or hypoxic conditions in Cos-7 cells, a 1.5 fold increase was obtained with the HIF-1 α and β constructs transfected independently and a 3.5 fold increase was noted in cells transfected with both constructs. Epo levels increased several fold in all Hep 3B cells that were incubated in hypoxic conditions. However, there was no additional increase in Epo levels in transfected Hep 3B cells. We therefore conclude that although the HIF-1 α and β subunits can independently co-transactivate the Epo enhancer, binding of both subunits and a hypoxic environment is necessary for maximal transactivation. Overexpression of the HIF-1 protein alone in normoxic or hypoxic conditions is insufficient for an increase in Epo secretion. Activation/inactivation and interaction of other tissue specific factors is necessary for an increase in Epo gene expression in response to hypoxia.

Keywords: co-transactivation, hypoxia, erythropoietin

INTRODUCTION

Erythropoietin (Epo) is a glycoprotein hormone that is responsible for maintaining a physiological level of circulating red blood cells (1,2). Epo is mainly synthesized by the adult

kidney (3). However, under conditions of severe anemia the adult liver is able to synthesize Epo (4). Epo secretion increases in response to hypoxemia. Several lines of evidence indicate that the regulation of Epo gene expression is primarily at the level of transcription (5,6).

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Hypoxia inducible factor 1 (HIF-1) is a transcription factor that is necessary for the transcriptional activation mediated by the erythropoietin gene enhancer in hypoxic cells (7). The HIF-1 protein is a heterodimer basic helix loop helix - PAS protein consisting of a 120 kDa HIF-1 α subunit complexed with a 91 to 94 kDa HIF-1 β subunit. Both subunits contact DNA in the major groove (8,9,10). The induction of HIF-1 DNA binding activity in hypoxic cells requires ongoing protein and RNA synthesis and protein phosphorylation (9). HIF-1 recognizes an 8 bp DNA motif 5' TACGTGCT-3' in the Epo enhancer (9). It is not known if overexpression of one or both HIF-1 subunits under normoxic or hypoxic conditions is sufficient to cause an increase in Epo production. HIF-1 is induced by hypoxia in various non-Epo producing cell types such as HeLa, a cervical carcinoma cell line, K293, a human embryonal kidney cell line and CHO, a Chinese hamster ovary cell line (8) and cos-7 (unpublished data). In addition, the Epo enhancer can be activated by hypoxia in non Epo producing cells (11). Semenza et al have shown that overexpression of HIF-1 in non-Epo producing cells can cause co-transactivation of the Epo enhancer in these cells. However it is not known if this overexpression can lead to Epo production in these cells. We performed co-transactivation experiments to assess the ability of the HIF-1 α and HIF-1 β cDNAs to transactivate a 38 bp Epo enhancer fragment bearing a HIF-1 binding motif, subcloned in a luciferase reporter plasmid. Experiments were performed in Hep 3B, a fetal hepatoma Epo producing cell line as well as in Cos-7, an SV40 transformed monkey kidney non-Epo producing cell line. Simultaneous transfection experiments were performed in normoxic (21%O₂) as well as in hypoxic (1%O₂) conditions. Our studies indicate that although HIF-1 α and β are able to independently activate the Epo enhancer in both Epo producing and non-Epo producing cells, both subunits are necessary for maximal activation. Overexpression of both HIF -1 subunits under normoxic or hypoxic conditions does not lead to

any increase in Epo production over baseline.

MATERIALS AND METHODS

Cell Culture

Hep 3B cells were grown in Eagles with Earles BSS medium containing 10% FCS, sodium pyruvate and non-essential amino acids and 1% Pen-Strep. Cells were divided and plated in 60 mm dishes 24 hours prior to transfection. Cos-7 cells were grown in Dulbeccos modified Eagles medium containing 10% FCS and 1% Pen-Strep. Cells were divided and plated 24 hours prior to transfection to be 70% confluent.

Plasmid Constructs

The full length human 3.4 kb HIF-1 α , obtained from G.L. Semenza, Johns Hopkin, MD was subcloned into pREP4, a protein expression vector containing the thymidine kinase promoter (Invitrogen, San Diego, CA). A 2.6 kb fragment containing the HIF1 β cDNA (nucleotides 46-2616) subcloned in pBM5/Neo (Invitrogen) was obtained from Oliver Hankinson, UCLA, CA.

We have previously described PEpoLuc, a luciferase reporter plasmid containing a 38 bp hypoxia inducible Epo enhancer fragment bearing the HIF-1 binding motif. The fragment is subcloned upstream of the Epo promoter (12).

Transfection

Hep 3B cells were transfected with HIF-1 α , HIF-1 β , HIF-1 α and HIF-1 β cDNAs, and pREP4 respectively. Each of the above constructs was co-transfected with PEpoLuc. Four 60 mm plates were transfected with each construct. For each plate 6 μ g of plasmid DNA, 2 μ g of pSVBgal and 4 μ g of PEpoLuc was used. In plates transfected with both HIF 1 α and β cDNAs, 3 μ g of HIF-1 α and 3 μ g of HIF-1 β was used. For each plate 50 μ g of Lipofectin was used for transfection. Cells were transfected as per the protocol from

GibcoBRL (Gaithersburg, MD). Media was changed 6 hours after transfection. Plates were incubated in 21% O₂, 5% CO₂, 74% N₂ or 1% O₂, 5% CO₂, 94% N₂ for 55 hours and then harvested. Luciferase activity was measured using a luminometer (LKB-1251). In order to correct for transfection efficiency a β -gal assay was performed on the cell lysate. The transfection experiments were repeated 3-8 times for each construct. Similar experiments were done in Cos-7 cells.

Erythropoietin Assay

Epo levels were measured in the supernate from Hep 3B cells by using an ELISA kit (Genzyme).

Statistical Analysis

The luciferase activity in lysates of cells transfected with different constructs or plasmid alone was determined in cells maintained in normoxia and hypoxia. The ratio of luciferase activity in cells transfected with the various cDNA constructs to activity in cells transfected with plasmid alone was determined. Each construct was tested 3-8 times in the transfection assay. The mean ratio and standard deviation for each construct was determined. A one tailed t-test was done to determine if the ratio for each construct was significantly greater than the control. A 2-tailed t-test was done to determine if the ratio of luciferase activity for construct/plasmid in cells transfected with either the HIF-1 α or HIF-1 β constructs was statistically different from the ratio in cells that were co-transfected with both the HIF-1 α and HIF-1 β cDNAs.

RESULTS

Hep 3B cells were co-transfected with PEpoLuc, a luciferase reporter plasmid containing a 38 bp Epo enhancer fragment subcloned upstream of the Epo promoter and

plasmids containing the HIF-1 α , HIF-1 β , HIF-1 α and 1 β cDNAs respectively. A control transfection was performed by co-transfecting PEpoLuc with pREP4, a plasmid vector without an insert (Invitrogen). Cells were incubated in normoxia or hypoxia for 55 hours. Media was aspirated and the Epo concentration was measured by ELISA. Cells were lysed and analysed for luciferase activity. In order to correct for a difference in transfection efficiency a β -gal assay was performed on the cell lysate. Each construct was tested 3-8 times. To determine the co-transactivation ability of each construct the ratio of the luciferase activity from each test construct to that from the control transfected cells was obtained. Similar experiments were performed in Cos-7 cells. Table 1 shows the results obtained from transfections in Hep 3B cells. Ratio of the luciferase activity in Hep 3B cells that were transfected with HIF-1 α and HIF-1 β alone and incubated in hypoxic conditions was 2.2 fold (p<0.005) and 3.4 fold (p<0.005) higher than that of the control transfected cells. However, a 6.9 fold (p<0.002) increase in activity was present in cells that had been transfected with both HIF-1 α and HIF-1 β . The luciferase activity in Hep 3B cells that were transfected with both HIF-1 α and HIF-1 β was significantly higher than in the cells that were transfected with either HIF-1 α cDNA (p<0.01) or HIF-1 β cDNA (p<0.02) independently. Ratio of luciferase activity in Hep 3B cells that had been maintained in normoxic conditions was 2 fold (p<0.1) with HIF-1 α , 3.2 fold (p<0.002) with HIF-1 β and 3 fold (p>0.005) with both HIF-1 α and β . However, the ratio of activity in cells co-transfected with both HIF-1 α and HIF-1 β was not significantly different from that in cells that were independently transfected with either the HIF-1 α (p.0.05) or HIF-1 β (p>0.05) constructs. It must be noted that although the overall ratios with the different constructs appear to be somewhat similar in the normoxic and hypoxic extracts, the luciferase activity in cells maintained in normoxia was extremely low. Ratio of luciferase activity in hypoxia/normoxia was 8-10 fold in all Hep 3B

cells irrespective of the constructs they were transfected with.

The results obtained from transfection in Cos-7 cells are shown in Table 2. The ratio of luciferase activity in transfected Cos-7 cells to that in cells transfected with vector alone under hypoxic conditions was 1.5 fold ($p < 0.02$) with HIF-1 α , 1.4 fold ($p < 0.005$) with HIF-1 β and 3.6 fold ($p < 0.025$) with HIF-1 α and HIF-1 β . Under normoxic conditions no increase was observed with HIF-1 α , a 1.3 fold ($p < 0.025$) increase with HIF-1 β and a 4.1 fold ($p > 0.05$) increase was observed with both HIF-1 α and β . However the difference in activity in cells transfected with both HIF-1 α and HIF-1 β constructs versus in cells transfected with either HIF-1 α or HIF-1 β alone was not statistically significant. The ratio of luciferase activity in hypoxia/normoxia in all

transfected Cos -7 cells was only 2-3 fold. Taken together these results show that although the HIF-1 α and HIF-1 β cDNAs are independently able to co-transactivate the Epo enhancer under normoxic or hypoxic conditions in Epo producing or non-Epo producing cells, both subunits of the HIF-1 protein are necessary for maximal co-transactivation. In addition, maximal co-transactivation occurs in Epo-producing cells and requires an hypoxic environment.

Serum Epo levels were measured in the media from Hep 3B cells. Results are shown in table 3. Epo levels were increased several fold in all plates that were maintained in hypoxia. However no additional increase was noted in cells that were transfected with the various HIF-1 constructs.

Table 1. Hep 3B cells were transfected with PEpoLuc and either pREP4, HIF-1 α , HIF-1 β or both HIF-1 α and HIF-1 β respectively. Cells were maintained in normoxia (21% O₂) or hypoxia (1% O₂). Cells were lysed and the luciferase activity was measured. The ratio of luciferase activity in cells transfected with pREP4, HIF-1 α , HIF-1 β or HIF-1 α and HIF-1 β to luciferase activity in cells that were transfected with pREP4 alone is reported below. The mean ratio \pm the standard deviation in cells maintained in normoxia as well as in hypoxia is shown. The p value for the one tailed t-test is shown.

Constructs	Normoxia luciferase units construct/pRE P4 n=4	Hypoxia luciferase units construct/pREP4 n=8
pREP4	1	1
HIF-1 α	2 \pm 1.5 ($p < 0.1$)	2.2 \pm 1.2 ($p < 0.005$)
HIF-1 β	3.2 \pm 0.6 ($p < 0.002$)	3.4 \pm 1.4* ($p < 0.005$)
HIF-1 α & HIF-1 β	3 \pm 0.7 ($p < 0.005$)	6.9 \pm 4.6* ($p < 0.002$)

* indicates that the p value for the 2 tailed t-test was < 0.05 .

Table 2. Cos-7 cells were transfected with PEpoLuc and either pREP4, HIF-1 α , HIF-1 β or both HIF-1 α and HIF-1 β respectively. Cells were maintained in normoxia (21% O₂) or hypoxia (1% O₂). Cells were lysed and the luciferase activity was measured. The ratio of luciferase activity in cells transfected with pREP4, HIF-1 α , HIF-1 β or HIF-1 α and HIF-1 β to luciferase activity in cells that were transfected with pREP4 alone is reported below. The mean ratio of activity \pm the standard deviation in cells maintained in normoxia as well as in hypoxia is shown. The p value for the one tailed t-test is shown below.

Constructs	Normoxia luciferase units construct/pREP4 n=3	Hypoxia luciferase units construct/pREP4 n=6
pREP4	1	1
HIF-1 α	0.9 \pm 0.1	1.5 \pm 1 (p<0.025)
HIF-1 β	1.3 \pm 0.5 (p<0.025)	1.4 \pm 0.9 (p<0.005)
HIF-1 α & HIF-1 β	4.1 \pm 2.8 (p<0.1)	3.6 \pm 3.2 (p<0.025)

* indicates that the p value for the 2 tailed t-test was <0.05.

Table 3. Hep 3B cells were transfected with pREP4, HIF-1 α , HIF-1 β , both HIF-1 α and HIF-1 β cDNAs respectively. Cells were plated at equal density and grown in 6 ml of media. Cells were incubated in normoxia (21% O₂) or hypoxia (1% O₂). Epo levels were measured in duplicate by an ELISA assay in the supernate from the transfected plates. The mean value \pm the standard deviation is shown in the table below.

Constructs	Normoxia Epo levels mU/ml n=3	Hypoxia Epo levels mU/ml n=5
pREP4	0.43 \pm 0.3	21.2 \pm 13.6
HIF-1 α	0.125 \pm 0.217	14 \pm 9.1
HIF-1 β	0.097 \pm 0.09	11.6 \pm 7.3
HIF-1 α & HIF-1 β	0.366 \pm 0.33	24.2 \pm 14.4

DISCUSSION

Epo is a hematopoietic growth factor whose synthesis and secretion increases in response to hypoxia. This increase is mainly regulated at the level of transcription (5,6). HIF-1 is a basic-helix-loop-helix -PAS transcription factor whose activity is induced by hypoxia (8). HIF-1 has been implicated in the transcriptional activation of several other genes in response to hypoxia such as the genes in the glycolytic pathway and the vascular endothelial growth factor (14,15). HIF-1 recognizes an 8 bp motif 5' TACGTGCT 3' in the 3' flanking sequence of the Epo gene. HIF-1 DNA binding activity is induced in response to hypoxia. This activity requires on going protein and RNA synthesis and protein phosphorylation (9).

There are several unanswered questions of biological significance regarding the relationship between hypoxia, Epo and the HIF-1 protein. Some of these are: Is there a cause and effect relationship between an increase in HIF-1 and an increase in Epo levels in response to hypoxia? Is an increase in HIF-1 levels alone in response to hypoxia sufficient to initiate a cascade of events leading to an increase in Epo levels?. Although HIF-1 DNA binding activity increases in response to hypoxia it is unclear if overexpression of the HIF-1 protein under normoxic conditions can cause transactivation of the Epo enhancer and an increase in Epo secretion. Semenza et al have shown that overexpression of both HIF-1 subunits in Hep 3B as well as in K293 cells can transactivate the Epo enhancer under normoxic or hypoxic conditions (16). Previous co-immunoprecipitation experiments have shown that HIF-1 α and HIF-1 β exist as a heterodimer in the absence of DNA (16). However it is not clear if overexpression of each subunit alone results in transcriptional activation.

Epo gene expression is inducible as well as tissue specific. HIF-1 DNA binding activity in response to hypoxia has been detected in several cell lines. Also, the Epo enhancer has been shown to mediate hypoxia inducible reporter gene

expression in non-Epo producing cells (11). It is likely that since HIF-1 is present in various non-Epo producing cell lines that an increase in HIF-1 levels alone in response to hypoxia is not sufficient to increase Epo levels. Transgenic mouse experiments indicate that the Epo gene utilizes multiple positive and negative regulatory elements to achieve cell type specific expression (17,18). Can an overexpression of HIF-1 override the effect of negative regulatory elements in and around the Epo gene and lead to Epo secretion by otherwise non-Epo producing cells?

Our current investigations have attempted to answer the above questions. We conducted our studies in Hep 3B cells, that produce Epo in a regulated fashion in response to hypoxia as well as in Cos-7 cells that do not produce Epo (19). Our results indicate that in Hep 3B cells HIF-1 α and HIF-1 β can independently transactivate the Epo enhancer under hypoxic conditions. However both subunits are necessary for maximal transactivation of the Epo enhancer. A similar result was obtained under normoxic conditions. However, since the expression of the reporter plasmid under normoxic conditions was very low it is difficult to conclude that the HIF-1 protein is able to transactivate the Epo enhancer under normoxic conditions. Therefore, HIF-1 binding and subsequent signal transduction requires a hypoxic environment. The above results are consistent with the hypothesis that hypoxic conditions lead to specific events such as protein phosphorylation that regulate HIF-1 DNA binding in response to hypoxia (9). Results of previously conducted transfection experiments in c4 cells, that lack HIF-1 β function have shown that HIF-1 β activity was necessary for induction of gene expression in response to hypoxia (19).

Similar transfection experiments performed in Cos-7 cells showed that the Epo enhancer was transactivated by overexpression of the various HIF-1 constructs under both normoxic or hypoxic conditions. Although reporter gene transcription appeared to be higher in Cos-7 cells co-transfected with both HIF-1 α and HIF-1 β cDNAs versus in cells that had been transfected with

HIF-1 α or HIF-1 β alone the difference in activity was not statistically significant. It is likely that tissue specific factors present in Epo-producing tissues are necessary for both maximal co-transactivation and Epo production. Previous experiments using a CAT reporter plasmid have shown 7-20 fold transactivation of the Epo enhancer in K293 cells under normoxic or hypoxic conditions respectively (16). This difference in activity compared to our results may be due the difference in the cell line used or the reporter plasmid. In addition our reporter plasmid contained a Epo promoter which would be expected to function better in Epo producing cells.

Functional activity was assessed by measuring Epo levels in transfected Hep 3B cells. We found an increase in Epo levels in all cells that had been maintained in hypoxia. However, no additional increase was seen in cells that had been transfected with the HIF-1 constructs. Western blots or HIF-1 binding assays were not performed to demonstrate an increase in HIF-1 protein as co-transfected cells showed an increase in reporter gene activity over baseline. There maybe several reasons for the above results. Prior transfection experiments have indicated that in order for transcriptional activation by HIF-1 to occur an as yet unidentified second factor needs to bind to the Epo enhancer downstream of the HIF-1 binding site (8). Although HIF-1 α and HIF-1 β RNA and protein levels have been reported to markedly increase in response to hypoxia (13), a similar increase in mRNA levels has not been observed by us (unpublished observation) or by others (19,20). Huang et al suggested that activation of HIF-1 depends on redox stabilization of the HIF-1 α subunit (21). Third, it is possible that the Epo gene is maximally upregulated in an hypoxic environment and that a further increase in HIF-1 protein is unable to elicit a larger effect.

Further experiments will need to be performed to elucidate other events that occur prior to transcriptional activation of the Epo gene and to identify tissue specific factors that cause

an increase in Epo secretion in response to hypoxia.

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