Hypoxia-inducible factor 1 (HIF-1) transactivates
the human leptin gene promoter

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Running title: Transactivation of the human leptin gene promoter by HIF-1
SUMMARY

Increased placental leptin has been demonstrated in preeclampsia, a pregnancy disorder associated with placental hypoxia. This suggests that leptin gene expression is enhanced in response to oxygen deficiency in this organ. In support of this hypothesis, we have previously shown that hypoxia activates the leptin promoter in trophoblast-derived BeWo cells. Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric HIF-1α / HIF-1β complex that regulates the transcription of hypoxia-responsive genes. To test whether this factor is involved in hypoxia-induced leptin promoter activation, BeWo cells were transiently transfected with a HIF-1α expression vector. Exogenous HIF-1α markedly increased luciferase reporter activity driven by the leptin promoter when HIF-1β was co-expressed in the same cells. This effect was similar to that elicited by CoCl₂, an agent known to stabilize endogenous HIF-1α. These data suggest that HIF-1α / HIF-1β dimers are involved in the effect of CoCl₂ to activate the leptin promoter. To confirm the implication of HIF-1, the cells were transfected with a dominant negative form of HIF-1α producing transcriptionally inactive HIF-1β / HIF-1α dimers. This mutant HIF-1α protein abolished CoCl₂ activation of the leptin promoter, providing direct evidence that the effect of CoCl₂ is mediated by endogenous HIF-1α. Deletion analysis and site-specific mutagenesis demonstrated that a HIF-1 consensus binding site (HRE) spanning –120 to –116 bp relative to start site, was required for CoCl₂ and exogenous HIF-1α induction of leptin promoter activity. Electrophoretic mobility shift assays performed with in vitro translated HIF-1α and HIF-1β proteins, demonstrated binding to this HRE and not to mutated sequences, only when both subunits were used together. These data demonstrate that leptin is a new hypoxia-inducible gene, which is stimulated in a placental cell line through HIF-1 interaction with a consensus HRE site located at –116 in the proximal promoter.
INTRODUCTION

Leptin, originally identified as a satiety factor secreted by adipose tissue, is also produced by the placenta in humans (1). Placental leptin mRNA (2) and protein (3) are markedly increased in preeclampsia, a disorder associated with maternal hypertension, reduction in placental blood flow and placental hypoxia (4). These observations have led to the proposal that the leptin gene could be induced by hypoxia. In support of this hypothesis, we have previously shown that gene expression and leptin release were increased in trophoblast-derived BeWo cells in response to various conditions of natural or chemical hypoxia. Moreover, the human leptin promoter was activated by hypoxia in these cells (5).

The hypoxia-inducible factor 1 (HIF-1) is a transcription factor of major importance in the cellular response to oxygen deficiency. HIF-1 comprises HIF-1α and HIF-1β subunits which both belong to the basic-loop-helix-PAS protein family (for review, see 6). The HIF-1β subunit is constitutively expressed. By contrast, HIF-1α is maintained at a low level in normoxic cells through proteasomal degradation of the protein. The von Hippel-Lindau tumor suppressor protein (pVHL) is a component of the complex which targets HIF-1α for polyubiquitination and degradation (7). Two recent observations indicate that pVHL binds to HIF-1α when a proline residue at codon 564 is hydroxylated (8, 9). Hydroxylation of HIF-1α is controlled by a Fe²⁺-dependent hydroxylase activity which is inhibited by decreased oxygen. This mechanism accounts for HIF-1α stabilization in hypoxic cells, allowing nuclear translocation and dimerization with HIF-1β. Stabilization of HIF-1α is also induced by chelating or substituting Fe²⁺ with desferrioxamine (DFO) and cobalt chloride (CoCl₂), respectively. This provides a molecular mechanism accounting for the ability of these agents to mimic the effect of hypoxia in experimental cell systems.

The present study was designed to test whether HIF-1 is involved in hypoxia-induced activation of the human leptin gene promoter in the placental BeWo cells. In order to investigate this, the transcriptional activity of HIF-1 was manipulated by overexpressing wild-
type or dominant negative form of HIF-1α. Our data provide evidence that induction of the leptin promoter activity by hypoxia is mediated by HIF-1, through a HIF-1 consensus binding site (HRE) located at –116 in the proximal promoter. This study adds the leptin gene to the list of hypoxia-inducible genes regulated by this transcription factor.
EXPERIMENTAL PROCEDURES

Cell culture - The human choriocarcinoma cell line, BeWo, was obtained through American Type Culture Collection (ATCC Rockville, MD, USA). The cells were cultured in RPMI 1640 medium (Gibco BRL, Gaithesburg, MD, USA) supplemented with 10% fetal calf serum and antibiotics in a humidified ambient atmosphere with 5% CO2 at 37°C.

Plasmids and constructs - 5’-deleted constructs containing various lengths of the human leptin promoter sequences upstream of the luciferase reporter gene have been described previously (5). Promoter fragments were referred according to their length in bp (p(bp)luc), relative to the transcription start site described in (10). Expression vectors encoding the wild-type HIF-1α (pcDNA3-HA-HIF-1α), a dominant negative form of HIF-1α (pcDNA3-HA-DN-HIF-1α) or wild-type HIF-1 β (pcDNA3-HA-HIF-1β) have been described in (11, 12).

Mutagenesis - A sequence contained within 0.146 kb of the leptin promoter sequence in the p(146)luc construct was mutated by using QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Two distinct mutations were generated. The motif 5’- GCACGTCG-3’ spanning -121 to -114 was replaced by 5’-GCTTAATG-3’ or by 5’-GCAAAACG-3’. The generated plasmids were designated p(146)lucmut1 and p(146)lucmut2, respectively. Each mutation was verified by direct sequencing and two plasmid preparations isolated from distinct clones were tested in transfection.

Transient transfection - LipofectAmine (Gibco BRL, Gaithesburg, MD, USA) mediated transfection of BeWo cells was performed as previously described (5). Briefly, one day prior to transfection, cells were plated into 35 mm 6 wells dishes and transfected with 500 ng/well of luciferase reporter construct and 60 ng/well of pRSV-β-galactosidase expression vector to normalize for transfection efficiency. In some experiments, expression vectors encoding HIF-1β, HIF-1α wild-type or HIF-1α mutant cDNA were co-transfected with the reporter constructs, as indicated in the figure legends. The total amount of transfected DNA was kept constant by adding the appropriate amount of pcDNA3 empty vector. Five hours post-
transfection, the medium was changed and the cells were cultured for 24 h in serum-free medium with or without addition of 100 µM of cobalt chloride (CoCl₂) (Sigma, St Louis, MO, USA). Transfections were performed at least in triplicate. In each experiment, individual data were calculated as the mean of triplicates and expressed as the ratio of luciferase to β-galactosidase activity measured in the same cell lysate, as previously described (5).

**Electrophoretic mobility shift assays** - HIF-1α and HIF-1β proteins were expressed by in vitro translation in rabbit reticulocyte lysates (Promega, Madison, WI). pcDNA3 empty vector, pcDNA3-HA-HIF-1α or pcDNA3-HA-HIF-1β were used as templates. Electrophoretic mobility shift assays (EMSA) were performed using a double-stranded probe encompassing a sequence contained within the first 0.146 kb of the human leptin gene promoter. The sense strand sequence of the wild-type (wt) oligonucleotide is 5’-GCTAGCAGCGCCCGCAGCTCGTACCCCTGAGGGGCG-3’. Two oligonucleotides containing distinct mutations of the underlined sequence in the wt probe, 5’-GCTAGCAGCGCCCGGCTTAATGCTACCCCTGAGGGGCG-3’ for mutant 1 (mut1) and 5’-GCTAGCAGCGCCCGGCAAACGCTACCCCTGAGGGGCG-3’ for mutant 2 (mut2) were also used as probes in EMSA. 32P-labeled oligonucleotides were generated by 5’ end labeling, using T4 polynucleotide kinase (New England Biolabs, Hitchin, UK) with [γ-32P]-ATP (50 µCi), and were gel purified. Reticulocyte lysates were incubated at room temperature in a 20 µL reaction containing 20 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM MgCl₂, 0.5 mM EDTA, 5 mM dithiothreitol, 5% glycerol (v/v) and 250 ng poly(dI/dC). One ng of 32P-labeled oligonucleotide (approximately 50,000 cpm / ng) was added 5 min later. Binding was then allowed to proceed for 15 min at room temperature. For competition experiments, 5-, 10- and 50-fold molar excess of unlabeled oligonucleotide, either wild-type or mutated, were added simultaneously with the labeled probe. DNA-protein complexes were resolved on native 5% polyacrylamide gels in 0.5X TBE (45 mM Tris-HCl pH 8.3, 45 mM boric acid and 1 mM EDTA). The gels were then dried and analyzed by autoradiography.
Statistical analysis - Statistical analysis was performed using Student’s t test for paired data, with significance defined as p< 0.05.
RESULTS

Effect of increasing HIF-1 transcriptional activity - An initial series of experiments was conducted to test whether increasing HIF-1 transcriptional activity would activate the leptin gene promoter in BeWo cells. Since transcriptional activation by HIF-1 depends on the amounts of HIF-1α available for heterodimerization with the constitutively expressed partner HIF-1β, we increased the cellular level of HIF-1α by the use of the pcDNA3-HA-HIF-1α expression vector. Previous experiments have shown that transient transfection of a fibroblast cell line with this plasmid allowed the detection of HIF-1α protein under normoxia (12). The effect of HIF-1α overexpression on the luciferase activity driven by 1.872 kb of the human leptin promoter was tested. As shown in Figure 1, overexpression of HIF-1α moderately increased (x 2-fold) luciferase activity compared to cells expressing the reporter only. However, when cells were co-transfected with HIF-1α and HIF-1β, reporter gene expression increased markedly compared to cells transfected with either subunit individually. CoCl₂ treatment induced a stimulatory effect on leptin promoter activity, which was closely similar to that elicited by HIF-1α overexpression both in the absence or presence of added HIF-1β. Moreover, when cells overexpressing HIF-1α were treated with CoCl₂, luciferase activity did not double, indicating that the effects of CoCl₂ and HIF-1α were not fully additive. These data support the implication of HIF-1α / HIF-1β dimers in the transactivation of the leptin gene promoter. Since overexpression of HIF-1β markedly enhanced luciferase activity in the presence of HIF-1α and/or CoCl₂, it is likely that low endogenous HIF-1β levels restrict HIF-1 transcriptional activity in these experimental conditions. Thus, in the next series of experiments, the cells were systematically transfected with the HIF-1β expression vector.

Deletion analysis of the 5'-flanking region of the leptin gene - To determine the promoter region mediating activation by exogenous HIF-1α or CoCl₂, BeWo cells were transfected with reporter constructs containing various lengths of the leptin gene promoter region. For each construct, the fold increase in luciferase activity elicited by either HIF-1α overexpression,
CoCl₂ treatment, or a combination of both was determined over non-stimulated cells. As mentioned above, HIF-1β was routinely transfected, whatever the experimental condition. A similar pattern of reporter gene expression was observed for several constructs containing up to 0.146 kb of the leptin gene 5'-flanking region (Figure 2). HIF-1α and CoCl₂ both individually stimulated luciferase activity by 5 to 7-fold. The effect of the two stimuli when combined, was always greater than that elicited by HIF-1α or CoCl₂ alone. However, when combined, these effects were never fully additive. In contrast to all other deleted constructs, the p(116)luc reporter vector was unresponsive to HIF-1α and/or CoCl₂. This analysis revealed that the first 146 bp of the leptin promoter harbor a sequence responsive to CoCl₂ and exogenous HIF-1α, which is missing or disrupted in the p(116)luc construct.

**Effect of a dominant negative form of HIF-1α** - To evaluate directly the involvement of HIF-1 in mediating the effect of CoCl₂ on this region of the leptin promoter, BeWo cells were co-transfected with the p(146)luc construct and a dominant negative form of HIF-1α (HIF-1α DN). This mutant HIF-1α heterodimerizes with HIF-1β but lacks a DNA binding domain, thereby producing transcriptionally inactive dimers (11). As shown in Figure 3, HIF-1α DN fully inhibited the stimulatory effect of exogenous HIF-1α on leptin promoter activity, demonstrating the efficiency of this dominant negative form of HIF-1α. HIF-1α DN also totally abolished the stimulatory effect of CoCl₂ on luciferase activity in cells transfected with the p(146)luc construct. By contrast, the reporter activity arising from the p(116)luc construct was not significantly affected. These data provide direct evidence that endogenous HIF-1α is required for CoCl₂-induced activation of the leptin promoter through a region extending 146 bp upstream of the transcription start site.

**Mutational analysis of a putative HRE within 0.146 kb of the leptin promoter** - Sequence analysis revealed the presence of a 5'-RCGTG-3' HIF-1 binding consensus sequence (HRE) within the 0.146 kb promoter fragment. This putative HRE, located between −120 and −116 on the non-coding strand, is disrupted in the p(116)luc construct. To assess its functional
importance, the sequence was mutated in the p(146)luc reporter vector. Constructs containing two distinct mutated fragments, p(146)lucmut1 and p(146)lucmut2, were transfected in BeWo cells and their capacity to respond to CoCl₂ treatment and HIF-1α overexpression was tested. As shown in Figure 4, the luciferase activity produced by both mutated leptin promoter fragments was not increased by these stimuli. This supports the hypothesis that this HRE consensus sequence is required for hypoxia-mediated induction of leptin promoter activity.

**In vitro binding of HIF-1α and HIF-1β to the –116 leptin promoter HRE** - To test whether the HRE identified within the proximal region of the human leptin promoter binds the HIF-1 complex composed of HIF-1α and HIF-1β, the two subunits were synthesized in vitro by using the reticulocyte lysate system. EMSA were performed with the proteins obtained in unprogrammed, HIF-1α or HIF-1β primed reticulocyte lysates. As shown in Figure 5, a specific complex with retarded migration appeared exclusively when HIF-1α and HIF-1β primed lysates were incubated together with the labeled probe containing the intact HRE. No complex was visualized when mutated oligonucleotides were used as the probe (Figure 5A). In addition, the specific binding of HIF-1α / HIF-1β to the wild-type probe was eliminated by competition with an excess of homologous unlabelled probe, but not with each of the two mutated oligonucleotides (Figure 5B). These data demonstrate that HIF-1 binds to the consensus HRE present within the proximal region of the leptin gene promoter.
DISCUSSION

We have shown previously that leptin gene expression is increased by hypoxia in a trophoblast-derived cell line (5). The present study provides functional evidence that HIF-1 mediates this effect via a HIF-1 responsive element located at −116 in the human leptin promoter. This conclusion is based on results obtained in experiments where the cellular level of HIF-1α was altered to induce or inhibit HIF-1 transcriptional activity. Consistent with the implication of HIF-1, exogenous overexpression of HIF-1α in the BeWo cells markedly activated the leptin promoter. Moreover, a similar amount of stimulation was produced by CoCl2 treatment, giving support to the idea that stabilization of endogenous HIF-1α by this agent mediates leptin promoter activation. The most compelling evidence for the implication of HIF-1 came from the use of a dominant negative form of HIF-1α, which totally abolished the effect of CoCl2. This demonstrates unequivocally that CoCl2-induced leptin promoter activity is driven by increased endogenous HIF-1α leading to the activation of HIF-1.

Sequence analysis reveals the presence of several putative HRE within the first 1.872 kb of the human leptin promoter. We have previously observed that two regions containing 1.87 kb and 1.20 kb of the promoter conferred respectively high and relatively lower responsiveness to hypoxia (5). These data suggested to us that a distal HRE located at -1.83 kb in the promoter could mediate the effect of hypoxia. However, this hypothesis was not confirmed by subsequent experiments performed in BeWo cells overexpressing HIF-1β. Indeed, we show here, that both CoCl2 and HIF-1α-induced activation of the leptin promoter is of a similar magnitude for each 5’- deleted fragment extending from 1.872 to 0.146 kb. Therefore, it is possible that distinct hypoxia responsiveness was coincidentally associated with promoter length, although it cannot be excluded that low levels of endogenous HIF-1β have been instrumental in this effect. In the present study, deletion analysis and site-specific mutagenesis clearly implicate the most proximal HRE of the leptin promoter in HIF-1 responsiveness.
These observations add the human leptin gene to a list of genes activated by hypoxia via the HIF-1 pathway. Following its initial discovery as a satiety factor, leptin has been subsequently implicated in a variety of functions, some of which are altered in response to decreased oxygen availability. For example, leptin has been shown to exert a potent pro-angiogenic effect in experimental systems in vitro and in vivo (13, 14). During preeclampsia, where placental hypoxia is a prominent feature, enhancing leptin production could be part of a compensatory response aimed at developing new vessels. Consistent with the idea that adipose leptin is also induced by hypoxia, we have recently observed that leptin gene expression is increased in human PAZ6 adipose cells in response to cellular hypoxia (15) and in the adipose tissue of rats submitted to hypobaric hypoxia\(^2\). If leptin also exerts a pro-angiogenic effect in this tissue, it can be anticipated that a local effect of leptin would be to stimulate vascularization during normal or pathological adipose tissue growth. Interestingly, the angiogenic capacity of adipose tissue has been used clinically to promote wound healing and revascularization of ischemic tissues (16, 17). This effect could be, at least in part, mediated by leptin, in concert with other angiogenic factors like VEGF (18). Besides the placenta, leptin is produced in several non-adipose tissues, including the stomach (19). Consistent with a stimulatory effect of a local hypoxic environment produced at wound sites, leptin gene expression is increased in gastric ulcers (20, 21). This suggests that leptin might participate in the mechanisms leading to ulcer healing in the stomach, since the hormone has been shown to promote skin wound re-epithelialization (22, 23). These observations favor the idea that up-regulation of leptin production by hypoxia is physiologically relevant not only in the placenta, but also in other leptin-producing tissues, including the adipose tissue.

Hypoxia is not the only condition that stabilizes HIF-1\(\alpha\) and activate HIF-1 transcriptional activity. Several hormones and growth factors, including insulin and insulin-like growth factor-1 (24), angiotensin II, thrombin and platelet-derived growth factor (11) and more recently, endothelin-1 (25) have been shown to increase the level of HIF-1\(\alpha\) in various cell types. In addition, inflammatory cytokines, such as IL-1\(\beta\) and TNF\(\alpha\), also induce HIF-1
activity in normoxic cells (26-28). It would be of interest to know whether these factors could regulate leptin gene expression in some cell-types, by activating hypoxia-independent HIF-1 pathways. Interestingly, this could be the case in the hypoxic placenta during preeclampsia, where increased inflammatory cytokine production has been described (29, 30).

In conclusion, the data presented here are consistent with the leptin gene being a genuine hypoxia-inducible gene. Moreover, they show that hypoxia mediates increased leptin gene expression \textit{via} HIF-1$\alpha$ and HIF-1-dependent transcriptional activity, as described for several other genes regulated by low oxygen availability.
REFERENCES


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FOOTNOTES

1 The abbreviations used are: HIF-1, hypoxia-inducible factor 1; pVHL, von Hippel-Lindau protein; DFO, desferrioxamine; CoCl₂, cobalt chloride; HRE, hypoxia response element; VEGF, vascular endothelial growth factor; TNFα, tumor necrosis factor α; IL-1β, interleukine 1β

FIGURE LEGENDS

FIGURE 1. Effect of overexpression of HIF-1α on leptin promoter activity. BeWo cells were transiently transfected with 500 ng of a luciferase reporter plasmid containing 1.872 kb of human leptin promoter (p(1872)luc) and 60 ng of a β-galactosidase expression vector. When indicated (+ HIF-1β), the cells were co-transfected with 1 µg of pcDNA3-HA-HIF-1β. Some cells were co-transfected with 1 µg of pcDNA3-HA-HIF-1α (dotted and black bars). Whatever the experimental condition, the total amount of transfected DNA was kept at 2 µg by addition of pcDNA3 empty vector. Five hours post-transfection, the medium was changed and the cells were cultured in serum free medium in the absence (open and dotted bars) or presence (hatched and black bars) of 100 µM CoCl₂, for 24 h. The cells were then lysed and luciferase and β-galactosidase activity were measured in cell lysate. Transfections were performed at least in triplicate and triplicate values were averaged to give the result of one experiment. Relative luciferase activity is the ratio of luciferase over β-galactosidase activity. Data are the mean ± SEM of 4 independent experiments.

FIGURE 2. Deletion analysis of the 5′-flanking region of the leptin gene. BeWo cells were transiently transfected with 500 ng of one of seven luciferase reporter constructs containing 5′-deleted leptin promoter fragments, 60 ng of a β-galactosidase expression vector, 1 µg of pcDNA3-HA-HIF-1β and either 1 µg of pcDNA3-HA-HIF-1α (dotted and black bars) or 1 µg of pcDNA3 empty vector (hatched bars). Five hours post-transfection, the medium was changed and cells were cultured in serum free medium in the absence (dotted bars) or presence (hatched and black bars) of 100 µM CoCl₂ for 24 h. Cells were then lysed and luciferase and β-galactosidase activity were measured in cell lysate. Transfections were performed at least in triplicate and triplicate values were averaged to give the result of one experiment. The results show the fold-increase in relative luciferase activity (ratio of
luciferase over β-galactosidase activity) over basal cells. Data are the mean ± SEM of 3 to 7 independent transfections.

**FIGURE 3. Effect of dominant negative form of HIF-1α on hypoxia-induced leptin promoter activity.** BeWo cells were transiently transfected with 60 ng of a β-galactosidase expression vector, 0.5 µg of pcDNA3-HA-HIF-1β and 500 ng of p(146)luc (A) or p(116)luc (B) luciferase reporter plasmid containing 0.146 kb or 0.116 kb of the leptin promoter, respectively. Cells were co-transfected with 4 µg of pcDNA3-HA-DN-HIF-1α (black bars) or 4 µg of pcDNA3 empty vector (open bars). 0.5 µg of pcDNA3-HA-HIF-1α was also added, when indicated (HIF-1α). The total amount of transfected DNA was kept at 5 µg by addition of pcDNA3 empty vector. Five hours post-transfection, the medium was changed and cells were cultured in serum free medium in the absence (Basal) or presence of 100 µM CoCl₂ for 24 h, as indicated. Cells were lysed and luciferase and β-galactosidase activity were measured. Transfections were performed at least in triplicate and individual values were averaged to give the result of one experiment. Relative luciferase activity was determined by the ratio of luciferase over β-galactosidase activity. Data are the mean ± SEM of 4 independent experiments. * P < 0.05 and *** P < 0.001 versus cells transfected with pcDNA3 empty vector.

**FIGURE 4. Effect of HRE site-specific mutation on hypoxia-induced leptin promoter activity.** BeWo cells were transiently transfected with 60 ng of a β-galactosidase expression vector, 1 µg of pcDNA3-HA-HIF-1β, 500 ng of one of the luciferase reporter plasmid p(146)luc (wt), p(146)lucmut₁ (mut1) or p(146)lucmut₂ (mut2) and either 1 µg of pcDNA3-HA-HIF-1α (dotted and black bars) or 1 µg of pcDNA3 empty vector (open and hatched bars). The wild-type promoter region encompassing –126 to –110 relative to transcription start site is shown, and the consensus HRE is boxed. The nucleotide changes in two distinct mutated sequences are underlined. Five hours post-transfection, the medium was changed and the
cells were cultured in serum free medium in the absence (open and dotted bars) or presence (hatched and black bars) of 100 µM CoCl₂ for 24 h. The cells were then lysed and luciferase and β-galactosidase activity were measured in cell lysate. Transfections were performed at least in triplicate and triplicate values were averaged to give the result of one experiment. Relative luciferase activity was determined by the ratio of luciferase over β-galactosidase activity. Data are the mean ± SEM of 4 independent experiments. * P < 0.05 and ** P < 0.01 versus basal cells.

FIGURE 5. In vitro binding of HIF-1α and HIF-1β on the HRE located at −116 in the leptin promoter. A: Radiolabeled oligonucleotides corresponding to wild-type (wt) or mutated (mut1 and mut2) HRE located at −116 in the leptin promoter were incubated with 2 µL of in vitro translated HIF-1α, 2 µL of in vitro translated HIF-1β, or both as indicated. Unprogrammed reticulocyte lysate was used as control and also to keep total volume of lysate at 4 µL. B: Competition assays were carried out by incubating the radiolabeled wt probe with in vitro translated HIF-1α and HIF-1β and cold wt or mutated probes in 5-, 10- or 50-fold molar excess, as indicated. These autoradiograms are representative of 4 independent EMSA.
Figure 1

Relative Luciferase Activity

- HIF-1β
- + HIF-1β

CoCl2
HIF-1α
Basal
HIF-1α + CoCl2
p(1872)luc

Relative Luciferase Activity

0 200 400 600 800 1000

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**Figure 2**

![Bar chart showing relative luciferase activity](image-url)

- **p(1872)luc**
- **p(1718)luc**
- **p(979)luc**
- **p(213)luc**
- **p(171)luc**
- **p(146)luc**
- **p(116)luc**

*Relative Luciferase Activity (Fold increase)*

Legend:
- HIF-1β + HIF-1α
- HIF-1β + CoCl₂
- HIF-1β + HIF-1α + CoCl₂
Figure 3

(A) p(146)luc

(B) p(116)luc

- HIF-1α DN
+ HIF-1α DN

Basal  HIF-1α  CoCl₂

Relative Luciferase Activity

*  ***
Figure 4

- Relative Luciferase Activity

wt 5'-GCCCGGCACGTGCCTAC-3'
3'-CGGGCCGTGCAACGATG-5'

mut2 5'-GCCCGGCAAAACGTGCCTAC-3'
3'-CGGGCCGTGCAACGATG-5'

mut1 5'-GCCCGGCAAATCGCTAC-3'
3'-CGGGCCGTGAACGATG-5'

p(146)luc

-126 -110

HIF-1β + CoCl₂
HIF-1β + HIF-1α
HIF-1β

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