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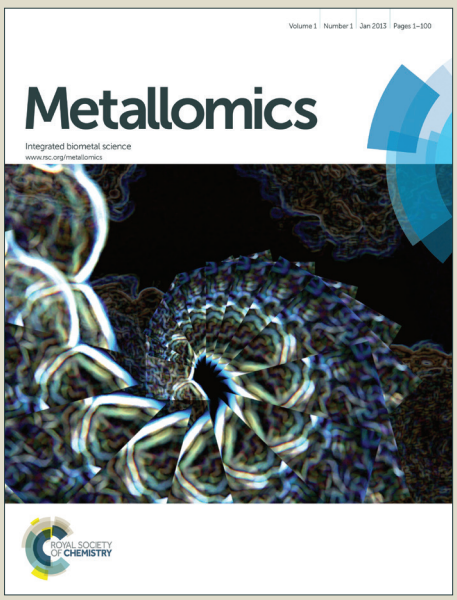

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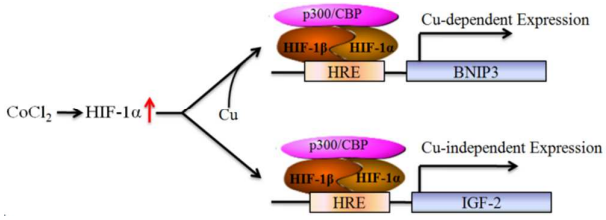
HIF-1 mediated expression of BNIP3 by cobalt chloride is Cu-dependent, but the expression of IGF-2 is Cu-independent.



Copper-dependent and –independent hypoxia-inducible factor-1 regulation of gene expression

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Running title: Copper and HIF-1 regulation of gene expression

ZZ and LQ made equal contributions to this study

This work was supported by National Science Foundation of China (grant number: 81230004 to Y. J. Kang)

Abstract

Hypoxia-inducible factor-1 (HIF-1) regulates the expression of vascular endothelial growth factor (VEGF), a process requiring copper (Cu) participation. HIF-1 is also involved in the expression of more than a hundred of genes, but it is unknown how HIF-1 differentially controls the expression of these genes timely and spatially. The present study was undertaken to test the hypothesis that Cu is not required for the expression of all HIF-1-regulated genes, thus exploring mechanistic insights into the differential control of multiple gene expression by one transcription factor. Human umbilical vein endothelial cells (HUVECs) were treated with siRNA targeting HIF-1α to define the essential role of HIF-1 in the regulation of BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) and insulin-like growth factor 2 (IGF-2) expression. A Cu chelator, tetraethelenepentamine (TEPA), was used to reduce intracellular availability of Cu. In comparison, a zinc chelator, N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN), was used to reduce intracellular zinc concentration. The expression of both BNIP3 and IGF-2 was completely suppressed in the HIF-1α deficient cells. The removal of Cu suppressed the expression of BNIP3, but did not affect that of IGF-2. The reduction of intracellular zinc did not cause the same effect. Further screening identified a group of genes whose expression required Cu and the others did not need Cu. The present study thus demonstrates Cu-dependent and –independent HIF-1 regulation of gene expression, indicating a mechanism for differential control of multiple gene expression by one transcription factor.

Keywords: Copper, zinc, HIF-1, BNIP3, IGF-2, HUVECs

Introduction

Hypoxia-inducible factor-1 (HIF-1) regulates the expression of multiple genes involved in cellular metabolism, homeostasis, and responses to stresses [1,2] . The regulation of HIF-1 transcriptional activity has been a major focus for understanding the fundamental process of the HIF-1 involvement in the cellular activities. HIF-1 is composed of HIF-1α and HIF-1β. The cellular stability of HIF-1α determines the ultimate activation of HIF-1. The level of HIF-1α is undetectable in most cell types under normoxic conditions due to its degradation by the ubiquitin-proteasome pathway, which is catalyzed by three HIF prolyl hydroxylases [3-5]. The hydroxylated HIF-1α is recognized by a von Hippel-Lindau protein (pVHL), which is a constituent of an ubiquitin ligase complex, targeting HIF-1α for degradation by proteasome in cytosol [6-9]. Under hypoxic conditions, HIF-1α escapes from the degradation pathway, accumulates in the cytosol, and translocates into nucleus, where it dimerizes with HIF-1β and interacts with cofactors to assemble the HIF-1 transcriptional complex, leading to transcriptional activation.

This general regulation of HIF-1 transcriptional activity has been implicated in all of the identified genes regulated by HIF-1. This raises a simple question: is the expression of all of the genes equally activated by HIF-1 at any given time and condition? It appears that not all of the genes controlled by HIF-1 are activated at the same time under a set of stress conditions, otherwise, the cellular activities regulated by HIF-1 would become coarsened. How is the expression of the genes differentially regulated by HIF-1 under varying conditions?

Cobalt inhibits prolyl hydroxylases leading to accumulation of HIF-1α and a concomitant increase in the transcriptional activity of HIF-1[2,10-12]. We have observed that cobalt-activated ex-

pression of vascular endothelial growth factor (VEGF) requires copper (Cu). The exposure of human umbilical vein endothelial cells (HUVECs) to cobalt chloride results in an accumulation of HIF-1α, and a concomitant increase in the expression of VEGF. The treatment of the cells with a Cu chelator, tetraethylenepentamine (TEPA), does not affect cobalt-induced accumulation of HIF-1α but blocks the increase in the VEGF mRNA and protein levels, an effect that is reversed by an addition of copper sulfate [13]. There are multiple genes that are activated by cobalt-induced accumulation of HIF-1α [14-16]. Does the expression of all of these genes require Cu? Answer to this question would help define a niche for understanding the differential regulation by HIF-1 of the expression of multiple genes, i.e., how does one transcription factor differentially regulates the expression of multiple genes timely and spatially?

In the present study, we tested the hypothesis that Cu is not required for the expression of all of the genes regulated by HIF-1. We used HUVECs to define Cu-dependent and –independent HIF-1 regulation of genes involved in different cellular activities; in which BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) is related to apoptotic cell death regulation and insulin-like growth factor 2 (IGF-2) is related to cell growth and metabolic activity. In addition, we screened 10 other genes whose expression is regulated by HIF-1 and distinguished Cu-dependent from Cu-independent expression of genes under the control of HIF-1.

Materials and Methods

Cell culture and treatment. Human umbilical vein endothelial cells (HUVECs, American Type Culture Collection, Virginia, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, California, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Auckland, NZ), 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were routinely maintained in a standard culture incubator with humidified air containing 5% CO_2 at 37°C. For treatment with reagents, cells were seeded in 6-well plates and grown overnight. Thereafter, $CoCl_2$ (Kelong, Chengdu, CN), tetraethylenepentamine (TEPA, Sigma, St. Louis, USA), N,N,N',N'-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN, Sigma, St. Louis, USA), or $CuSO_4$ (Kelong, Chengdu, CN) was added to the cultures, followed by further incubation for a period of time indicated. Cells numbers were counted by a hemocytometer.

Measurement of intracellular copper and Zinc. Cell samples were digested with nitric acid (AR). Digests were colourless or light yellow and clear with no visible precipitate or residue. Ultrapure water was added to each vessel to dilute the digests to a proper concentration for subsequent analyses of copper or zinc. Cu or zinc concentrations were determined by graphite furnace atomic absorption spectrophotometry (ICE3250; Thermo Scientific).

Western blotting analysis of proteins. Protein extracts were obtained after lysing cells in the RIPA lysis buffer (Beyotime, Jiangsu, CN) containing 1% complete EDTA-free protease inhibitor cocktail (Roche, Mannhein, DE) for 30 min on ice. Equal loading of protein was assured by prior quantitation using the Bio-Rad assay. An appropriate amount of protein in total cell lysates was resolved in a SDS-polyacrylamide electrophoresis gel and transferred onto a polyvi-

nylidene difluoride membrane (Bio-RAD, USA). Membranes were blocked for 1 hr in Tris-buffered saline/Tween 20 (TBST) (10 mM Tris-HCl, pH 8.0, 125 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk and incubated overnight at 4°C with the following primary antibodies diluted in blocking buffer: mouse anti–human HIF-1α monoclonal antibody (1:250，BD Biosciences, San Jose, USA), and mouse anti-beta Actin monoclonal antibody (1:500, ZSGB-BIO, Beijing, CN). After washing with TBST, the membranes were incubated with a horseradish peroxidase-linked anti-mouse or anti-rabbit IgG antibody (1:500, ZSGB-BIO, Beijing, CN) diluted in TBST for 1 hr at room temperature. Proteins were visualized using a chemiluminescence HRP substrate (Millipore, Billerica, USA).

Real-time quantitative RT-PCR. Total RNAs were extracted from HUVECs harvested after various treatments using an RNAiso Plus method. Two steps real-time PCR was performed in this study. The reverse transcription (RT) reaction was carried out using 900 ng of total RNA following the protocol of M-MLV RTase cDNA Synthesis Kit (TaKaRa, Biotechnology, Dalian, CN). A primer optimization step was tested for each set of primers to determine the optimal primer concentrations. Once the optimal primer concentration was determined, 1.0 μl primers, 10 μl SYBR Green Master Mix (TaKaRa, Biotechnology, Dalian, CN) and 20–50 ng of cDNA sample were applied to a total volume of 20 μl PCR amplification. Reactions were run on a Multicolor Real-time PCR Detection system (Bio-Rad, California, U.S.A), under the following cycling conditions: denaturation at 95℃ (30 sec); 45 cycles at 95℃ (5 sec) and 60℃ (20 sec). Cycle threshold (Ct) values were obtained from the Bio-Rad CFX Manager software. Ribosomal protein S18 (RPS18) was also determined for each RNA sample as control. Fold change of relative

mRNA expression was determined using the $2^{-\Delta\Delta CT}$ method. The primer sets used for RT-PCR are listed.

RPS18	Forward-TTCGGAACTGAGGCCATGAT	Reverse-TTTCGCTCTGGTCCGTCTTG
IGF2	Forward-GACCGCGGCTTCTACTTCAG	Reverse-AAGAACTTGCCCACGGGGTAT
BNIP3	Forward-TCAGCATGAGGAACACGAGCGT	Reverse-GAGGTTGTCAGACGCCTTCCAA
VEGF	Forward-TTGCCTTGCTGCTCTACCTCCA	Reverse-GATGGCAGTAGCTGCGCTGATA
PGK-1	Forward-CTCCGCTTTCATGTAGAGGAAG	Reverse-GACATCTCCTAGTTTGGACAGTG
GLUT1	Forward-CTGAAGTCGCACAGTGAATA	Reverse-TGGGTGGAGTTAATGGAGTA
LDH	Forward-ACCCAGTTTCCACCATGATT	Reverse-CCCAAAATGCAAGGAACACT
GAPDH	Forward-AACTTCTCCTGGTCTCTCSGCT	Reverse-GCAAATGCTTTAAGGAAGAAG
SDF-1	Forward-ACCGCGCTCTGCCTCA	Reverse-CATGGCTTTCGAAGAATCGG
PAI-1	Forward-CAGACCAAGAGCCTCTCCAC	Reverse-ATCACTTGGCCCATGAAAAG
EDN1	Forward- TCCTGCTCTTCCCTGATGGA	Reverse- TGCTCAGGAGTGTTGACCCA

Gene silencing of HIF-1α. The siRNA targeting human HIF-1α and mismatched control were designed and synthesized from RiboBio (Guangzhou, China). The siRNA sequences for HIF-1α were as follows: sense, GGCCUCUGUGAUGAGGCUUtt; antisense, AA-GCCUCACAGAGGCCtt. The optimal transfection efficiency was determined from our preliminary study testing the range from 10-100 nM, and we selected the condition that the siRNA caused an optimal silencing effect with minimal cytotoxicity. After HUVECs were transfected with 25 nM annealed siRNA targeting human HIF-1α or negative mismatched control siRNA in serum-free and antibiotics-free media, a Lipofectamine 2000 reagent (Invitrogen, California, U.S.A) was used as the transfection reagent according to the manufacturer's instruction. After 36 hrs transfection, cells were trypsinized and collected for further analysis as described in experimental procedure.

Statistical analysis. Data were initially evaluated using one-way ANOVA, followed by Dunnett t test. $p < 0.05$ was considered significant.

Results

Gene silencing of HIF-1α suppressed the expression of BNIP3 and IGF-2 in response to cobalt treatment

The treatment of HUVECs with 50 μM $CoCl_2$ in cultures for 12 hrs caused a significant increase in the protein level of HIF-1α, as determined by western blotting analysis, shown in Fig 1A and 1B. Corresponding to the cellular accumulation of HIF-1α, the mRNA levels of BNIP3 and IGF-2 were significantly increased (Fig 1C and 1D). The treatment of HUVECs with siRNA targeting HIF-1α blocked cobalt-induced accumulation of HIF-1α and suppressed the expression of BNIP3 and IGF-2 (Fig 1).

Cu chelation by TEPA suppressed cobalt-induced BNIP3 but not IGF-2 expression

To define the requirement of Cu for Co-induced gene expression, a Cu chelator TEPA was used. The HUVECs were treated with 50 μM $CoCl_2$ for 6 hrs, then some cobalt-treated cells were exposed to 25 μM TEPA for additional 6 hrs. As negative or positive controls, HUVECs in cultures were respectively treated with the same volume of saline for 12 hrs or with 50 μM $CoCl_2$ for 12 hrs. The results showed that addition of 25 μM TEPA in cultures reduced intracellular Cu levels (Fig 2A) and had little effect on cobalt-induced accumulation of HIF-1α (Fig 2C and 2D). This treatment completely inhibited cobalt-induced BNIP3 expression, but did not suppress IGF-2 expression (Fig 2E and 2F).

Zinc chelation by TPEN did not affect cobalt-induced BNIP3 or IGF-2 expression

In comparison with the effect of Cu reduction, a zinc chelator TPEN was used to reduce intracellular zinc levels. The HUVECs were treated with 10 μM TPEN for 6 hrs after they were

exposed to 50 μM $CoCl_2$ for 6 hrs. This TPEN treatment reduced intracellular zinc levels (Fig 2B), and had no effect on cobalt-induced accumulation of HIF-1α (Fig 2C and 2D). In addition, this TPEN treatment affected neither BNIP3 expression nor IGF-2 expression induced by cobalt (Fig 2E and 2F).

Cu-dependent and –independent HIF-1 regulation of gene expression

To further define Cu dependency or independency of HIF-1 regulation of gene expression, we screened the expression of other 8 different genes under the treatment with cobalt chloride for 12 hs. The result presented in Fig 3 showed that vascular endothelial growth factor (VEGF), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glucose transporter 1 (GLUT1), and phosphoglycerate kinase 1 (PGK1), required Cu for expression and lactate dehydrogenase (LDH), endothelin 1 (EDN1), stromal-derived factor-1 (SDF-1), serpin E1(PAI-1) did not need Cu for expression.

Discussion

Previous studies demonstrated that both hypoxia- and cobalt-induced VEGF expression through activation of HIF-1 transcription factor are Cu-dependent [13,17]. HIF-1 regulates the expression of multiple genes and many intrinsic and extrinsic stress factors activate HIF-1 transcriptional activity [18,19]. It appears that under different stress conditions, a different set of genes are activated to participate in the cellular response. Therefore, although the same HIF-1 regulates a multiple genes, the expression of genes differs under a given condition. How does HIF-1 differentially regulate the expression of genes under one condition from the other? There must be additional regulatory mechanisms that modulate the transcriptional activity of HIF-1, so that the expression of different set of genes at a given time or condition is activated and the others remain silence. In the present study, a Cu-dependent and –independent HIF-1 regulation of gene expression were identified, providing a mechanistic insight into the differential regulation of gene expression under the control of one transcription factor, HIF-1.

Cobalt up-regulates the expression of several genes[16,20], among which HIF-1 activation plays a vital role [21,22], but others are not mediated by HIF-1 transcription factor [16]. To define Cu-dependent and –independent HIF-1 regulation of gene expression, we selected two different genes that regulates different cellular activities and whose expression is activated under different stress conditions; BNIP3 is involved in the anti-apoptotic activity and IGF-2 regulates cellular mitotic and metabolic responses. We first determined whether their expression under the stimulation of cobalt chloride is dependent on HIF-1 activation. Gene silencing of HIF-1α significantly suppressed cobalt-induced accumulation of HIF-1α and completely blocked the expression of

BNIP3 and IGF-2, confirming that cobalt-induced expression of both BNIP3 and IGF-2 is HIF-1 dependent.

TEPA has been shown to enter cells and be able to reduce both Cu levels and Cu,Zn-superoxide dismutase activity in a time dependent manner without affecting cell viability or cell differentiation[23]. On the other hand, TPEN also enters cells but reduces intracellular zinc levels [24]. In previous studies, we found TEPA at the concentration used in the present study (25 μM) does not cause any significant changes in cellular metabolism and function in the cultured HUVECs[13]. In the present study, we found TEPA reduced intracellular Cu levels and TPEN decreased zinc levels. In the presence of TEPA, cobalt-induced BNIP3 expression was completely suppressed, but the expression of IGF-2 was not affected. In contrast, in the presence of TPEN, the expression of BNIP3 or IGF-2 was not affected, indicting the suppressed expression of BNIP3 is Cu reduction-specific.

A concern may be raised in the use of TEPA as Cu chelator, i.e., it may also chelate cobalt. However, this possibility was excluded from this and our previous study[13]: First, the cells were treated with $CoCl_2$ for 2 hrs before exposed to TEPA, at which time HIF-1α had been accumulated to the highest level in the cells. Second, the expression of both BNIP3 and IGF-2 induced by cobalt depends on the accumulation of HIF-1α, but only the expression of BNIP3 was suppressed by TEPA, indicating a specific effect. Third, a concomitant addition of equal molar concentration of copper chloride blocked the inhibitory effect of TEPA on HIF-1 regulated gene expression [13], confirming the specific Cu chelation effect. Therefore, the HIF-1 mediated expression of BNIP3 is Cu-dependent, but the expression of IGF-2 is Cu-independent.

We further screened the expression of other 8 genes that are known to be regulated by HIF-1 to define the Cu dependency. In this screening, we found genes whose expression was not activated by cobalt chloride, and the genes whose expression was activated by cobalt chloride but either requiring or not requiring the presence of Cu. This result further demonstrates Cu-dependent and –independent HIF-1 regulation of gene expression. This is an important finding. As stated above, differential regulation of multiple genes by one transcription factor is a fundamental question. Under a specific condition, only a particular set of genes are up-regulated; others remain silence although they are under the control of the same transcription factor. In the present study, the requirement of Cu for the expression of some HIF-1 regulated genes provides a new insight into the differential regulation by HIF-1 of gene expression. However, it will be an important undertaking in the future to probe how Cu manipulates HIF-1 regulation of the expression of different genes.

In summary, the present study provided a novel insight into the differential regulation of expression of genes under the control of HIF-1 through the analysis of Cu-dependency and –independency. This Cu manipulation of HIF-1 regulation of gene expression would reflect a general understanding of gene expression regulated by transcription factors. Although one transcription factor controls the expression of multiple genes, the up-regulation of a set of genes in response to specific conditions is additionally controlled by accessory factors besides the transcription factor. In this context, Cu participation in the HIF-1 regulation of gene expression distinguishes a specific set of genes from the other, and Cu depletion specifically suppresses the expression of some, but not all HIF-1 regulated genes.

Acknowledgments

This work was supported by National Science Foundation of China (grant number: 81230004 to Y. J. Kang) and Sichuan University West China Hospital. The funding sources had no influence in study design; in the collection, analysis and interpretation of data; in the writing of the report; and in the decision to submit the article for publication.

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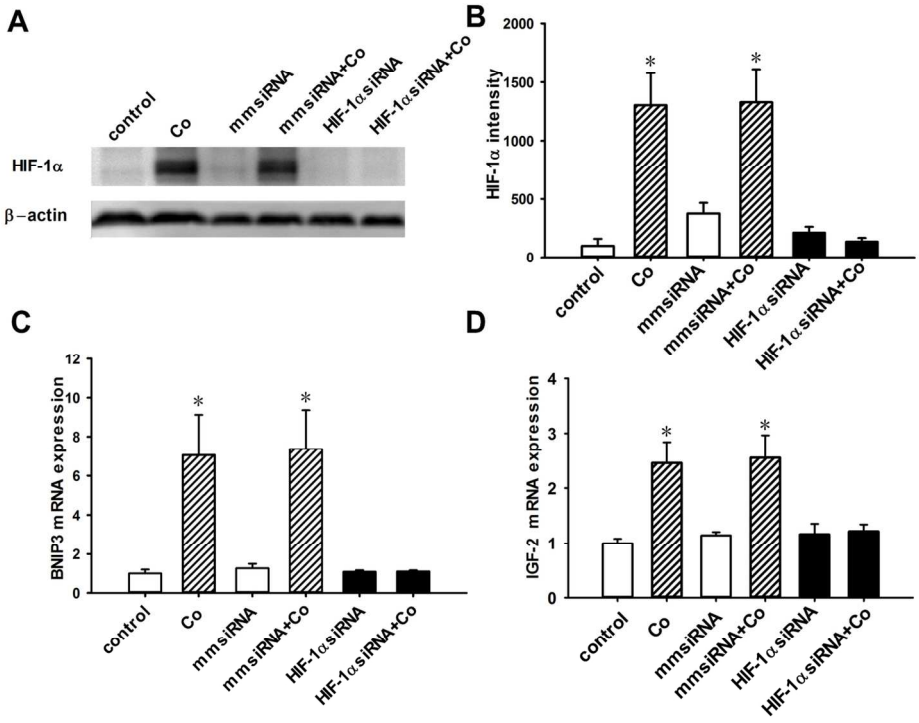
Figure Legends

Fig 1. Effects of HIF-1α gene silencing on the expression of BNIP3 and IGF-2. A. Effects of $CoCl_2$ and HIF-1α gene silencing on HIF-1α accumulation detected by western blotting. HUVECs were transfected with HIF-1α siRNA or mismatched siRNA (mmsiRNA) for 36 hrs before the treatment with 50 μM $CoCl_2$ (*lane 2,4,6*) for 12 hrs. **B**. Semi-quantitative analysis of HIF-1α accumulation detected by western blotting. **C.** Effects of HIF-1α gene silencing on the level of mRNA for BNIP3 detected by a real-time RT-PCR analysis. The treatment protocol was as the same as described for A. **D.** Effects of HIF-1α gene silencing on the level of mRNA for IGF-2 detected by a real-time RT-PCR analysis. All of the data were obtained from three independent experiments. *, significantly different from untreated controls ($P < 0.05$).

Fig 2. TEPA or TPEN on cobalt-induced BNIP3 and IGF-2 expression. A. Intracellular Cu concentrations after treatment with 25 μM TEPA or 10 μM TPEN for 6 hrs in combination with 50 μM $CoCl_2$ treatment for 12 hrs. **B.** Intracellular zinc concentrations under the same treatment above. **C.** Western blot analysis of HUVECs treated for 12 hrs with 50 μM $CoCl_2$ only (*lane 2*) or for 12 hrs with 25 μM TEPA only (*lane 3*), or with 50 μM $CoCl_2$ for 6 hrs first, then for additional 6 hrs with 25 μM TEPA (*lane 4*). In addition, cells were treated with 10 μM TPEN only (*lane 5*), or with 50 μM $CoCl_2$ for 6 hrs first, then for additional 6 hrs with 10 μM TPEN (*lane 6*). **D.** Semi-quantitative analysis of HIF-1α accumulation detected by western blotting. **E**. Effects of $CoCl_2$, TEPA, or TPEN on the level of mRNA for BNIP3 detected by a real-time RT-PCR analysis. **F**. Effects of $CoCl_2$, TEPA, or TPEN on the level of mRNA for IGF-2 detected by a real-time

RT-PCR analysis. All of the data were collected from three independent experiments and expressed as mean ± SEM. *, significantly different from untreated controls ($P < 0.05$).

Fig 3. Cu-dependent and –independent HIF-1 regulation of gene expression. HUVECs treated for 12 hrs with 50 μM $CoCl_2$ only or with 25 μM TEPA only, or for 6 hrs with 50 μM $CoCl_2$ first, then for additional 6 hrs with a concomitant presence of 25 μM TEPA. All of the data were collected from three independent experiments and expressed as mean ± SEM. *, significantly different from untreated controls ($P < 0.05$).



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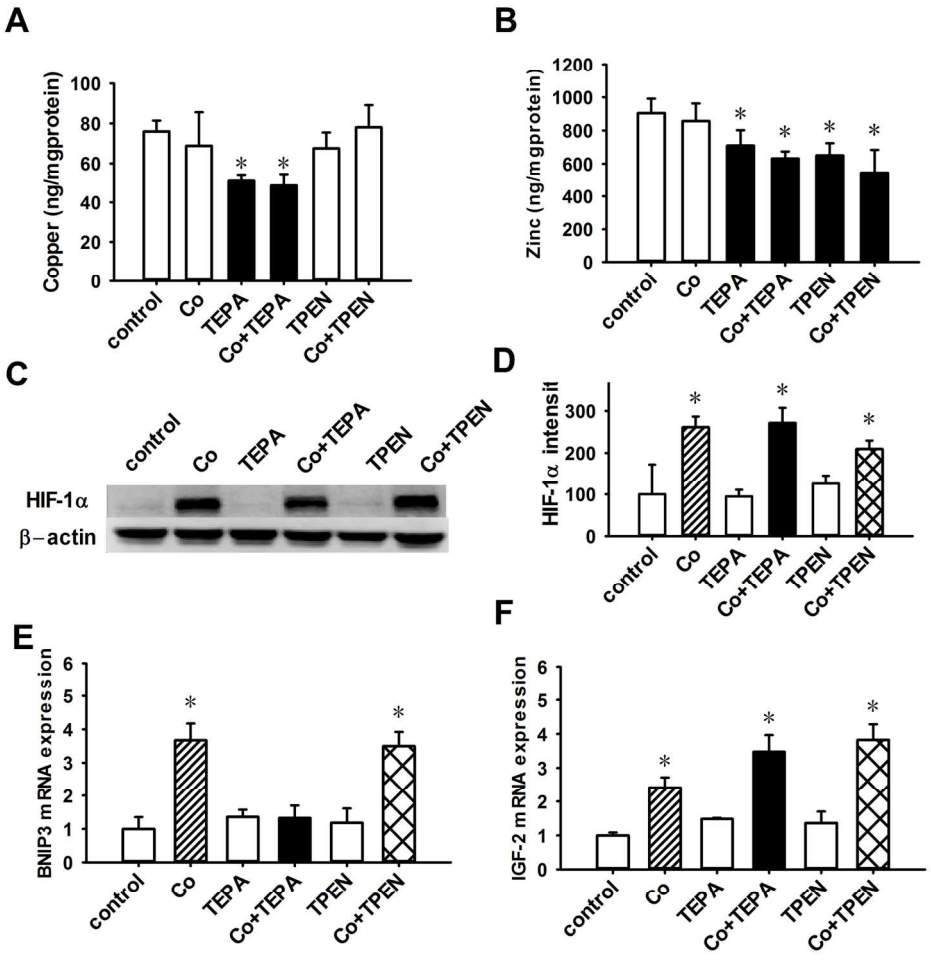

TEPA or TPEN on cobalt-induced BNIP3 and IGF-2 expression.
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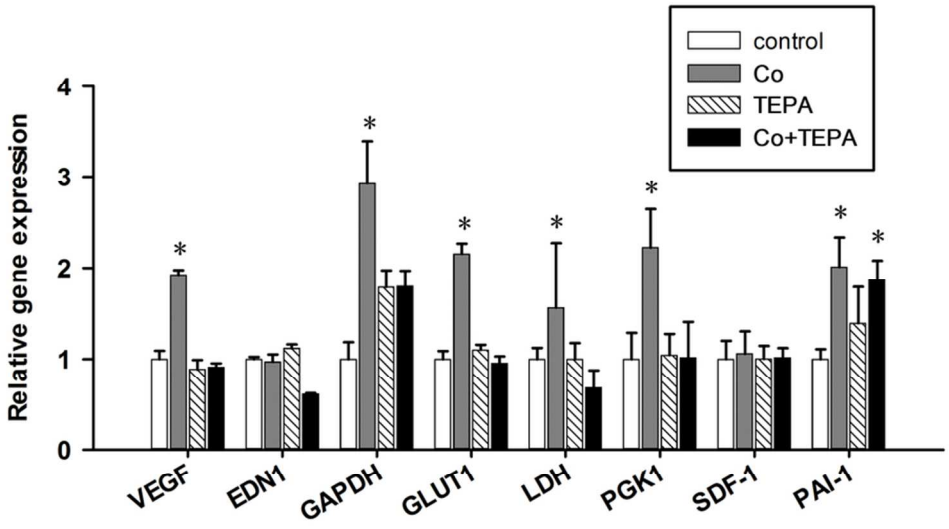

Cu-dependent and –independent HIF-1 regulation of gene expression.
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