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Copper regulation of hypoxia-inducible factor-1 activity

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ABBREVIATIONS LIST

TEPA, tetraethylenepentamine; HIF, hypoxia-inducible factor; HRE, hypoxia responsive element; DFO, deferoxamine; FIH-1, factor inhibiting HIF-1;CCS, Cu chaperone for superoxide dismutase -1; IGF, insulin-like growth factor; PHD, HIF prolyl hydroxylase; EMSA, electrophoretic mobility shift assay; VEGF, vascular endothelial growth factor

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ABSTRACT

Previous studies have demonstrated that copper (Cu) up-regulates hypoxia-inducible factor 1 (HIF-1). The present study was undertaken to test the hypothesis that Cu is required for HIF-1 activation. Treatment of HepG2 cells with a Cu chelator tetraethylenepentamine (TEPA) or siRNA targeting Cu chaperone for superoxide dismutase 1 (CCS) suppressed hypoxia-induced activation of HIF-1. Addition of excess Cu to relieved the suppression by TEPA, but not that by CCS gene silencing, indicating the requirement of Cu for activation of HIF-1, which is CCS-dependent. Cu deprivation did not affect production or stability of HIF-1α, but reduced HIF-1α binding to the hypoxia-responsive element (HRE) of target genes and to p300, a component of HIF-1 transcriptional complex. Cu likely inhibits factor inhibiting HIF-1 (FIH-1) to ensure the formation of HIF-1 transcriptional complex. This study thus defines that Cu is required for HIF-1 activation through regulation of HIF-1α binding to the HRE and the formation of HIF-1 transcriptional complex.

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Previous studies have shown that pressure overload causes copper (Cu) decrease in the heart in association with an inhibition of myocardial angiogenesis and of transition from cardiac hypertrophy to heart failure in a mouse model (Jiang et al., 2007). Dietary supplementation with physiologically relevant levels of Cu replenishes cardiac Cu and reverses hypertrophic cardiomyopathy in the presence of pressure overload induced by ascending aortic constriction in the mouse model (Jiang et al., 2007). Further studies have shown that dietary Cu supplementation increases myocardial vascular endothelial growth factor (VEGF) levels along with an enhanced angiogenesis. Importantly, systemic administration of anti-VEGF antibody blunts Cu regression of hypertrophic cardiomyopathy (Jiang et al., 2007). It has also been shown that dietary Cu restriction causes suppression of VEGF expression in the heart and Cu replenishment recovers myocardial VEGF expression (Elsherif et al., 2004). In addition, Cu stimulates VEGF expression in cultured human keratinocytes, and enhances angiogenesis and promotes wound healing (Sen et al., 2002).

In an attempt to understand molecular mechanisms by which Cu stimulates VEGF expression, we have studied the role of transcription factor, hypoxia-inducible factor 1 (HIF-1), in regulation of Cu-mediated VEGF expression. Cu chelation suppresses the transcription activity of HIF-1 and gene silencing of α subunit of HIF-1 (HIF-1α) blocks Cu stimulation of VEGF expression (Jiang et al., 2007). Other studies have shown that excess Cu stabilizes HIF-1α and stimulates VEGF expression under both normoxic and hypoxic conditions (Martin et al., 2005). However, it is important to know how Cu regulates HIF-1 transcription activity.

HIF-1 is composed of HIF-1α and HIF-1β (Wang et al., 1995). The synthesis and accumulation of HIF-1α is a rate-limiting step for activation of HIF-1 (Huang et al., 1998;Wang and Semenza, 1993b) .The expression level of HIF-1α is undetectable in most cell types under normoxic conditions due to its degradation by the ubiquitin-proteasome pathway, which is

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mediated by proline hydroxylation of HIF-1α catalyzed by three HIF prolyl hydroxylases (PHDs) (Huang et al., 1998;Jaakkola et al., 2001;Ivan et al., 2001). The hydroxylated HIF-1α is recognized by a von Hippel-Lindau protein (pVHL), which is a constitute of an ubiquitin ligase complex, targeting HIF-1α subunit for degradation by proteasome in cytosol (Maxwell et al., 1999;Masson et al., 2001;Ohh et al., 2000;Tanimoto et al., 2000). Under hypoxic conditions, HIF-1α escapes from the degradation pathway, accumulates, and translocates into nucleus, where it dimerizes with HIF-1β and interacts with cofactors to assemble the HIF-1 transcriptional complex. Some transition metals such as cobalt and nickel enhance HIF-1 transcription activity by stabilizing HIF-1α protein (Hirsila et al., 2005;Ke et al., 2005;Maxwell and Salnikow, 2004;Yuan et al., 2003). In the nucleus, HIF-1α is hydroxylated on its C-terminal asparagine by another dioxygenase, factor inhibiting HIF-1 (FIH-1) (Mahon et al., 2001). FIH-1 prevents the interaction between HIF-1α and its cofactors, such as p300, and inhibits the transcription activity of HIF-1 (Lando et al., 2002a;Lando et al., 2002b;McNeill et al., 2002;Freedman et al., 2002;Dames et al., 2002). FIH-1 is an iron dependent enzyme. Therefore, iron chelation enhances HIF-1 transcription activity (Wang and Semenza, 1993a).

Cu is capable of stabilizing HIF-1α by a mechanism involving the inhibition of prolyl hydroxylases (Martin et al., 2005;van Heerden et al., 2004). However, this action of Cu would be as the same as other transition metals such as cobalt and nickel, which are not essential for HIF-1 activation, but enhance HIF-1 activity when the cells are exposed to excess amount of these transition metals (Hirsila et al., 2005;Ke et al., 2005;Maxwell and Salnikow, 2004;Yuan et al., 2003). The critical question that we are addressing is whether or not Cu is essential for HIF-1 transcription activity.

Our previous studies have suggested that Cu is required for HIF-1 transcription activity. Cu

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chelation in cultured cells blocks insulin-like growth factor-1 (IGF-1)-induced HIF-1 binding to hypoxia responsive element (HRE) and VEGF expression. This inhibitory effect can be relieved by addition of excess Cu in cultures (Jiang et al., 2007). In addition, we have found that this Cu action depends on a Cu chaperone for superoxide dismutase 1 (CCS) and CCS gene silencing blocks IGF-1-induced HIF-1 HRE binding and VEGF expression, mimicking the effect of Cu chelation. Furthermore, CCS directly interacts with HIF-1α, as revealed by an immunoprecipitation assay (Jiang et al., 2007).

There are multiple sites that potentially require Cu for activation of HIF-1, including HIF-1α synthesis, stabilization, translocation from cytosol to nucleus, binding to the HRE sequence of target genes, and HIF-1 transcriptional complex formation. In the present study, we report that Cu is required for HIF-1 binding to the HRE sequence of target genes, and for the HIF-1 transcriptional complex formation in a HepG2 human hepatoma cell line.

MATERIAL AND METHODS

Cell Culture and Treatments. Human hepatoma cell line HepG2 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were routinely cultured in 95% air and 5% CO_2 at 37 ºC. Hypoxic conditions were introduced by incubating cells in a tightly sealed chamber maintained at 1% O_2, 5% CO_2 and balanced with N_2 at 37 ºC. For treatment with reagents, cells were seeded in culture dishes and grown overnight. Thereafter, tetraethylenepentamine (TEPA) or $CuSO_4$ was added to the cultures, followed by further incubation for the time periods as indicated in each experiment. In some experiments, deferoxamine (DFO) was added at 16 h prior to cell harvesting to induce HIF-1α accumulation.

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RNA Interference. siRNAs targeting human FIH-1 and CCS, and a negative mismatched control were designed and synthesized by Ambion, Inc. (Austin , TX). The siRNA sequences for FIH-1 were: sense; GCUUAUUGAGAAUGAGGAGtt and antisense; CUCCUCAUUCUCAAUAAGCtc. The siRNA sequences for CCS were: sense, GGACCAGAUGGUCUUGGUAtt, and antisense, UACCAAGACCAUCUGGUCCtt. After monolayer cultures reached 50% confluence, the cells were transfected with 100 nM FIH-1, CCS or negative mismatched siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Antibiotics were added to the medium 24 h after transfection and cells were used for experimental procedures 48 h after transfection.

Nuclear Extract Preparation. Nuclear extracts were prepared as described previously (Hellwig-Burgel et al., 1999) with minor modifications. Briefly, cells were washed once on dish with ice-cold phosphate-buffered saline (PBS). Ice-cold buffer A (10 mM Tris-HCl, pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl) containing freshly added 0.4 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM dithiolthreitol (DTT) and 1% protease inhibitor cocktail (Sigma) was overlaid on cells in the dish and incubated for 10 min. The cells were then harvested by scraping with a rubber cell policeman and lyzed by Dounce homogenization. Nuclei were pelleted by centrifugation and then resuspended in ice-cold buffer B (20 mM Tris-HCl, pH 7.8, 420 mM KCl, 1.5 mM $MgCl_2$, 20% glycerol) containing freshly added 0.4 mM PMSF, 0.5 mM DTT, 1% protease inhibitor cocktail and 1 mM Na_3VO_4 and incubated for 30 min on ice with occasional taping. The extracts were clarified by centrifugation at 12,000 x g for 15 min at 4 ºC, aliquoted, and stored at -80 °C.

Quantitative analysis of VEGF. VEGF levels were determined using a Quantikine Human VEGF Immunoassay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction.

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Western Blotting. Whole cell lysates were prepared as previously described (Jaakkola et al., 2001). Appropriate amount of protein in total cell lysates were resolved in a SDS-PAGE gel and transferred onto a polyvinylidene difluoride membrane (Whatman, Sanford, ME). Membranes were blocked for 1 h in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) containing 5% non-fat dry milk and incubated overnight at 4 ºC with the following primary antibodies diluted in blocking buffer: HIF-1α (BD Biosciences, San Jose, CA); p300, FIH-1 and CCS (Santa Cruz Biotech, Santa Cruz, CA), and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) (StressGen, Victoria, Canada). After washing with TBST, the membranes were incubated with a horseradish peroxidase-linked anti-mouse or -rabbit IgG antibody (GE Health Carr, Piscataway, NJ) for 1 h at room temperature. Proteins were visualized using an ECL system (GE Health Care).

Immunoprecipitation. Immunoprecipitation was performed using anti-HIF-1α or anti-p300 (BD Biosciences) antibodies as described previously (Feng et al., 2005). The immunopreciptates were subjected to Western blotting analysis as described above.

Transient Transfection and Luciferae Reporter Assay. HepG2 cells were transfected with a luciferase reporter gene construct pH3SVL plasmid (a kind gift of Prof. RH Wenger) by Lipofectamine 2000 according to the instruction provided by the manufacturer (Invitrogen). After recovering, cells were treated with different reagents as described in each experiment. Subsequently, cells were incubated for 16 h under hypoxia. In some experiments, cells were treated with DFO for induction of HIF-1α. After washing with PBS, cells were lyzed with a passive lysis buffer provided by Promega (Madison, WI). Luciferase activities were determined using a luciferase assay system (Promega) and normalized to total cellular protein. In some experiments, pH3SVL luciferase reporter plasmid was co-transfected with siRNAs, as described

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above.

Electrophoretic Mobility Shift Assay. Double-stranded oligonucleotides containing the HIF-1 binding sequence from erythropoietin (EPO) HRE (5′-TCT GTA CGT CAC ACT CAC CTC-3′) (Santa Cruz Biotechnology), were end-labeled with [γ-^{32}P]ATP (GE Health Care) using T4-polynucleotide kinase (Promega). Unincorporated nucleotide was removed by gel-filtration using a Nick column (GE Health Care). The γ-^{32}P-labeled HRE probe was incubated with 5-10 μg of nuclear extract and 1 μg of poly (dI-dC)·(dI-dC) (GE Health Care) in a buffer containing 10 mM Tris-HCl, pH 7.8, 20 mM KCl, 1 mM EDTA, 5 mM DTT, 1 mM MgCl2, and 5% glycerol overnight at 4 °C in a total volume of 20 μl. For competition assay, a 200-fold molar excess of unlabeled HRE was added to the reaction prior to addition of labelled probe. For supershift assay, 2 μl of mouse monoclonal antibody against HIF-1α (BD Biosciences) was added to the reaction 2 h before electrophoresis. Electrophoresis was performed using 5% nondenaturing polyacrylamide gel at 200 V in 0.5 x Tris-buffered EDTA for 2 h at 4 °C. The gels were dried and subjected to autoradiography for visualization.

Immunocytochemistry. HepG2 cells grown on a chamber slide were incubated under normoxia or hypoxia with or without additional treatments. After incubation, media were removed, and cells were fixed with 4% paraformaldehyde. The fixed cells were washed three times with PBS plus 3% bovine serum albumin, and incubated at 4°C overnight with rabbit polyclonal anti-HIF-1 antibody (BD Biosciences). Then the cells were washed three times with PBS plus 3% bovine serum albumin, and incubated with FITC-conjugated goat anti-rabbit IgG antibody (Sigma) for 1 h. Reaction omitting the primary antibody served as a negative control.

Statistical Analysis. Data were evaluated using Student's *t* test. $p < 0.05$ were considered to be significant.

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RESULTS

Cu Deprivation Suppresses HIF-1 Transcription Activity. To define the essential role of Cu in activation of HIF-1 in HepG2 cells in response to hypoxia, we treated cultured HepG2 cells with a Cu chelator, TEPA, to decrease intracellular levels of labile Cu. The efficacy of TEPA in reducing cellular Cu was determined by the effect of TEPA on accumulation of a Cu-sensitive CCS in the cells. CCS levels increase when Cu levels decrease, which have been used as an indicator of Cu depletion (Prohaska et al., 2003). The result presented in Fig. 1A shows that the accumulation of CCS in HepG2 cells increased after the cells were treated with 50 μM TEPA for 24 h, and further increased after the treatment for 72 h, indicating that TEPA treatment effectively decreased cellular Cu levels. Under this experimental condition, TEPA up to a final concentration of 100 μM in the cultures did not cause cytotoxicity, as evaluated by a lactate dehydrogenase release assay (data not shown).

The effect of Cu deprivation on HIF-1 transcription activity was evaluated by two different assays. The first, hypoxia-induced VEGF production in cultured HepG2 cells was determined, and the second, a HIF-1-specific reporter assay was performed. In the first experiment, the presence of 50 μM $CuSO_4$ in cultures enhanced hypoxia-induced VEGF production, but the presence of 50 μM TEPA suppressed the production. To confirm that the TEPA-suppressed VEGF production results from Cu deprivation, varying concentrations of $CuSO_4$ were added to the TEPA-treated cultures and a Cu concentration-dependent recovery of TEPA suppression of VEGF production was observed (Fig. 1B), indicating a selective effect of Cu chelation by TEPA.

We next determined the effect of Cu deprivation by TEPA on HIF-1 transcription activity using a luciferase reporter assay. The HepG2 cells were transfected with a reporter plasmid containing 6X HIF-1 binding site fused with a luciferase gene (Wanner et al., 2000). As expected,

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the luciferase reporter gene was activated by hypoxia and TEPA treatment significantly reduced the luciferase activity. This effect of TEPA on luciferase activity was also relieved by addition of excess Cu in a Cu concentration-dependent manner (Fig. 1C), further demonstrating the selective effect of Cu deprivation on HIF-1 transcription activity.

To further verify the effect of Cu on HIF-1 transcription activity, we determined the effect of CCS gene silencing on HIF-1-specific HRE binding activity. We have shown previously that CCS is required for Cu activation of HIF-1 (Jiang et al., 2007). Therefore, CCS gene silencing should block hypoxia-induced activation of HIF-1, but addition of excess Cu should not overcome this effect because the action of Cu is mediated by CCS. The data presented in Fig. 1D show that a siRNA targeting CCS effectively blocked CCS production in these cells and suppressed hypoxia-induced activation of HIF-1. Addition of excess Cu did not relieve the inhibitory effect of CCS-silencing on HIF-1 activation.

Cu Deprivation Affects neither Production nor Stability of HIF-1α. To define the action site at which Cu is required for activation of HIF-1 transcription activity, we examined whether or not Cu chelation by TEPA affects HIF-1α protein expression or stability. Under normoxic conditions, HIF-1α protein undergoes a degradation process mediated by hydroxylation of the proline residues 402 and 564 of HIF-1α by PHDs. HepG2 cells were treated with or without 100 μM TEPA under normoxic conditions for 24 h and subsequently cultured for 5 h under hypoxic conditions (1% O_2). Hypoxia-induced HIF-1α accumulation was detectable 5 min and reached a peak level 5 h after hypoxia (data not shown). After hypoxic treatment for 5 h, the cells were exposed to normoxic conditions for varying periods of time. There were no differences between the control and TEPA-treated cultures in hypoxia-induced HIF-1α production. Upon re-oxygenation, the HIF-1α protein was rapidly decreased in a time-dependent manner and

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become undetectable 20 min after re-oxygenation. TEPA treatment did not affect HIF-1α protein degradation (Fig. 2A). It was noted that when the cells were re-oxygenated for 2 min, higher molecular weight bands appeared, most likely representing the ubiquitinated HIF-1α. The data presented here thus demonstrate that Cu deprivation by TEPA affects neither expression nor stability of HIF-1α.

Cytosolic accumulation of HIF-1α results in its nuclear translocation. To determine if TEPA treatment affects HIF-1α nuclear localization, immunohistochemistry was performed on HepG2 cells using an anti-HIF-1α antibody (Fig. 2B). Under normoxic conditions, HIF-1α protein was undetectable. Hypoxia induced a remarkable accumulation of HIF-1α in the nucleus. TEPA treatment changed neither HIF-1 protein levels nor localization. Addition of $CuSO_4$ to TEPA treated cells did not affect HIF-1 protein levels or localization either (data not shown).

Cu Deprivation Decreases HIF-1 Binding to the HRE. Since Cu deprivation did not change expression or stability of HIF-1α, we continued our effort to further define possible action site for Cu in activation of HIF-1. We examined the DNA-binding activity of HIF-1 using nuclear extracts from TEPA-treated HepG2 cells by an electrophoretic mobility shift assay (EMSA). The result presented in Fig. 3A shows that hypoxia increased the binding of HIF-1 to HRE (lane 2). The treatment with TEPA decreased the binding intensity (Fig. 3A, lane 6). To confirm the specificity of HIF-1 binding to the DNA sequence, a competition assay using 200-fold unlabeled HRE and a supershift assay using a monoclonal anti-HIF-1α antibody were performed. Unlabeled HRE competed off the binding of the labeled HRE to HIF-1 (Fig. 3A, lane 3), and the antibody shifted the DNA-protein complex to a higher molecular mass band (Fig. 3A, lane 4&5) , indicating the specific binding of HIF-1 to the HRE (Fig. 3A). Addition of Cu to TEPA-treated cells relieved the inhibition of HRE binding by TEPA, as shown in Fig. 3C.

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Cu Deprivation Reduces HIF-1 Transcriptional Complex Formation. An important mechanism for regulation of HIF-1 transcription activity is HIF-1 transcriptional complex formation. Among the cofactors involved in the formation of HIF-1 transcriptional complex, p300 is essential for HIF-1 transcription activity. We examined whether or not Cu deprivation by TEPA affects the interaction between HIF-1 and p300. We used an anti-HIF-1α antibody to perform an immunoprecipitation assay. Under hypoxic condition, p300 was co-immunoprecipitated with anti-HIF-1α antibody (Fig. 4A). However, the amount of p300 that was precipitated by the anti-HIF-1α antibody was dramatically decreased under the treatment with TEPA (Fig. 4A). Addition of $CuSO_4$ to the TEPA-treated cells elevated the amount of p300 protein precipitated by anti-HIF-1α antibody (Fig. 4A). A retro-immunoprecipitation was also performed using anti-p300 antibody to confirm the HIF-1α/p300 interaction. As shown in Fig. 4B, TEPA treatment decreased the amount of HIF-1α protein precipitated by anti-p300 antibody and addition of $CuSO_4$ relieved this inhibition.

Under normoxic conditions, FIH-1 hydroxylates Asn803 residue in HIF-1α and therefore inhibits the binding of HIF-1α to p300. However, we found that Cu deprivation did not affect the level of FIH-1 (Fig. 5A). We then determined if Cu inhibits the activity of FIH-1. If Cu inhibits FIH-1 activity, TEPA should relieve this inhibition leading to recovery of FIH-1 activity and enhance the inhibition of HIF-1α binding to p300 along with a suppression of HIF-1 transcription activity. In this context, if FIH-1 activity is inhibited by a different mechanism, Cu deprivation should not suppress the HIF-1 transcription activity. To test this hypothesis, we used a siRNA targeting FIH-1 to delete FIH-1 in HepG2 cells (Fig. 5A), and then examined the effect of TEPA on HIF-1α transcription activity under FIH-1 deletion condition. The results presented in Figs. 5B and 5C show that deletion of FIH-1 made TEPA treatment ineffective in suppression of

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HIF-1 transcription activity, suggesting that FIH-1 is a possible target of Cu action.

To further define the role of Cu in FIH-1 activity, we determined the effect of Cu deprivation on iron chelation-induced activation of HIF-1. It has been shown that iron is required for the activity of FIH-1 and iron chelation results in an inhibition of FIH-1, leading to activation of HIF-1. If the action of Cu is mediated by inhibition of FIH-1, the activation of HIF-1 by iron chelation should not be affected by TEPA treatment because iron chelation-induced FIH-1 inhibition cannot be recovered by Cu deprivation. We used an iron chelator, DFO, which has been shown to induce HIF-1 activation through inhibition of FIH-1 (Hirsila et al., 2005). The treatment with DFO increased HIF-1 transcription activity in HepG2 cells, as determined by VEGF expression (Fig. 6A) and luciferase reporter assay (Fig. 6B). Pretreatment with TEPA did not affect DFO-induced VEGF expression or luciferase reporter activation (Fig. 6).

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DISCUSSION

In the present study, we report that Cu deprivation suppresses hypoxia-induced activation of HIF-1, thus inhibits the expression of genes such as VEGF controlled by this transcription factor. We have presented several pieces of evidence that demonstrates Cu is required for HIF-1 activation. In the presence of Cu chelator TEPA, the expression of VEGF and the luciferase reporter activity were suppressed under hypoxic conditions. This suppression was relieved by addition of excess Cu to cell cultures, indicating the selective effect of Cu chelation by TEPA and the important role of Cu in HIF-1α activation. The action of Cu is mediated by CCS, as indicated by the facts that CCS gene silencing suppressed HIF-1 activty and that addition of excess Cu did not relieve this suppression. These results thus demonstrate that Cu is required for HIF-1 activation. The important question is how Cu is involved in HIF-1 activation.

The essential role of Cu in HIF-1 activation is different from that of excess Cu-enhanced HIF-1 transcription activity. The activation of HIF-1 under hypoxic conditions is proceeded by two distinct processes. The level of the α subunit of HIF-1 is an important determining factor for HIF-1 activation, and it is controlled by pVHL-associated protein degradation mediated by PHDs. Under hypoxic conditions, this pathway is inhibited so that HIF-1α accumulates in the cell. In the nucleus, the transactivity of HIF-1 is regulated by HIF-1 binding to HRE and HIF-1 transcriptional complex formation, which is regulated by FIH-1. Exposure to excess Cu results in an accumulation of HIF-1α in cells (Martin et al., 2005;van Heerden et al., 2004). Other transition metals such as nickel and cobalt have also been shown to activate HIF-1 through increasing the accumulation of HIF-1α in cells. This activation of HIF-1 by transition metals including Cu is mediated by increasing the stability of HIF-1α by inhibiting the degradation process (Hirsila et al., 2005;Salnikow et al., 2004;Martin et al., 2005;Ke et al., 2005;Maxwell and Salnikow, 2004;Yuan

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et al., 2003).

Hypoxia is a major regulatory mechanism of HIF-1 activity. Hypoxia stabilizes HIF-1α protein, but has no effect on HIF-1α transcription, which has been shown by unchanged HIF-1α mRNA levels under hypoxic conditions (Wenger et al., 1997). HIF-1α is also regulated by an mTOR-dependent pathway (Hudson et al., 2002), which enhances HIF-1α protein expression (Treins et al., 2002;Phillips et al., 2005;Laughner et al., 2001). However, Cu deprivation affected neither synthesis nor stability of HIF-1α, indicating that Cu is required at different steps of HIF-1 activation.

The present study provides important insights into the mechanism of action of Cu in the process of HIF-1 activation. First, Cu may be required for HIF-1 binding to HRE in the target genes. In previous studies, we have observed that Cu deprivation decreases the binding of HIF-1 to HRE in cultured cells in response to IGF-1 stimulation as determined by an ELISA assay, but the total level of HIF-1α in cells was not reduced by Cu deprivation (Jiang et al., 2007). In the present study, we observed that under hypoxic conditions, Cu deprivation also decreased the binding of HIF-1 to HRE, but we used a different assay, EMSA, to determine the binding. Therefore, these complementary results obtained from different cells under different experimental conditions confirm the inhibitory effect of Cu deprivation on the binding of HIF-1 to HRE. It appears that this process is Cu chaperone CCS-dependent. It has been shown that CCS is present in the nucleus (Casareno et al., 1998) and we have also observed the appearance of CCS in the nucleus by an immunocytochemical procedure (data not shown). While CCS may be important for Cu transport into the nucleus, it is apparently required for Cu interaction with HIF-1α because CCS is co-immunoprecipitated with HIF-1α (Jiang et al., 2007) and CCS gene silencing suppresses HIF-1 activty.

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Second, Cu apparently plays an important role in the formation of HIF-1 transcriptional complex. The formation of HIF-1 transcriptional complex is a critical step for HIF-1 activation of target gene expression. HIF-1α enters into nucleus and associates with HIF-1β to assemble HIF-1. Although the binding of HIF-1 to HRE is important, the initiation of the transcription of target genes requires a transcriptional complex (Kallio et al., 1998;Roth et al., 2004). This complex includes several proteins and among these proteins is a p300 (Forsythe et al., 1996;Kallio et al., 1998;Arany et al., 1996;Carrero et al., 2000;Gray et al., 2005). In this process, a critical regulatory factor is FIH-1, which causes hydroxylation of the Asn803 of HIF-1α to inhibit the recruitment of cofactors including p300 to HIF-1 transcriptional complex. It appears that this is a process involving the action of Cu, as indicated by the result that Cu deprivation reduced the binding of HIF-1 to p300. This result suggests that either Cu is required for the binding of HIF-1 to p300 or Cu inhibits FIH-1 activity, relieving the binding capacity of HIF-1 to its cofactors.

The results here suggest that Cu is likely involved in the cofactor-recruiting step of activation of HIF-1 through inhibition of FIH-1 activity. We have observed that FIH-1 gene silencing using RNA interference technique blocked the inhibitory effect of Cu deprivation by TEPA on hypoxia-induced luciferase reporter activity. To further confirm the possibility of Cu regulation of HIF-1 transcription activity through its action on FIH-1, we examined the effect of Cu deprivation on iron chelation-induced activation of HIF-1. It has been shown that FIH-1 activity is iron-dependent. In previous studies, it has been suggested that PHDs are iron-dependent so that iron chelation can inhibit the activity of PHDs, leading to an accumulation of HIF-1α (Wang and Semenza, 1993a). However, recent studies have shown that iron chelation by DFO mainly inhibited FIH-1 activity (Hirsila et al., 2005). If Cu acts through its inhibition of FIH-1 activity, Cu deprivation should not affect iron-chelation-induced HIF-1 activation. This indeed

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was observed in the present study and the result suggests that Cu inhibition of FIH-1 activity is likely involved in the regulation of HIF-1 transcription activity.

A recent study showed that a Cu, Zn chelator, Clioquinol, increased functional HIF-1α protein, leading to the target gene expression (Choi et al., 2006). Of interest, Clioquinol inhibited ubiquitination of HIF-1α in a Cu(II)- and Zn(II)-dependent manner, but it prevented FIH-1 from hydroxylation of the asparagine residue (803) in a Cu(II)- and Zn(II)-independent fashion (Choi et al., 2006). This implies that Clioquinol inhibition of FIH-1 activity was not by the chelation of metals, but by an unknown mechanism. The logK_i (K_i: binding constant) values of the Cu chelator TEPA are 23.1 for Cu(II) and 15.3 for Zn(II) (Smith and Martell, 1976). The logK_i values of Clioquinol are 15.8 for Cu(II) and 12.5 for Zn(II) (Cherny et al., 1999). Apparently, K_i value of TEPA-Cu is about 7 orders of magnitude higher than that of Clioquinol-Cu ($K_{i\ \text{TEPA-Cu}}/K_{i\ \text{Clioquinol-Cu}}$). TEPA also has a remarkable higher selectivity for Cu(II) over Zn(II) ($K_{i\ TEPA\text{-}Cu}/K_{i\ TEPA\text{-}Zn}$: about 8 orders of magnitude) than Clioquinol ($K_{i\ \text{Clioquinol-Cu}}/K_{i\ \text{Clioquinol-Zn}}$: about 3 orders of magnitude). In addition, Zn has been shown to be a negative regulator of HIF-1 activation (Chun et al., 2000). Taken together, the discrepancy between TEPA and Clioquinol in the regulation of FIH-1 may be, at least in part, due to a remarkable difference in Cu selectivity and affinity.

It is important to note that TEPA is also able to bind other transition metals, such as Fe, Co and Ni, but with much lower affinity compared to Cu (Smith and Martell, 1976). Iron Chelation inactivates PHD and causes HIF-1α accumulation and activation (Fig 6). Although previous studies showed that exogenous Co and Ni treatment caused HIF-1 accumulation and activation (Maxwell and Salnikow, 2004), there is yet no evidence that shows the effect of Co or Ni chelation on HIF-1 activation. The physiological concentrations of Co and Ni are very low and TEPA chealtion of Co or Ni is unlikely to affect HIF-1 activation.

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In summary, we show here that Cu chelation suppresses hypoxia-induced HIF-1 transactivity and VEGF production in HepG2 cells. The requirement of Cu for HIF-1 activation and for the target gene expression provides a novel insight into the mechanism by which Cu manipulation affects various clinical practices, in particular, when angiogenesis is concerned. In this study, we have identified two action sites of Cu in HIF-1α activation (Fig 7), but cannot exclude other possibilities. First, Cu is likely required for the binding of HIF-1 to the HRE sequence of target genes, a process that requires Cu chaperone CCS. Second, Cu apparently is involved in the regulation of HIF-1 transcriptional complex formation. In this regard, Cu may inhibit the activity of FIH-1 to retain the capacity of HIF-1α binding to its cofactors. However, Cu deprivation does not affect expression or stability of HIF-1α, indicating that these processes are not Cu dependent although excess Cu can stabilize HIF-1α through a mechanism shared by other transition metals that are not essential for HIF-1 activation. Although more detailed mechanistic insights are required to provide a comprehensive understanding of Cu requirement for HIF-1 activation, the present study sheds the light for Cu manipulation of cancer treatment and cardiovascular diseases in clinical practice.

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FOOTNOTES:

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FIGURE LEGENDS

Figure 1. **Effects of TEPA and siRNA targeting CCS on HIF-1 activation.** (A), Effects of TEPA treatment on CCS levels. Cells were incubated in the presence or absence of 50 μM TEPA for 24 h or 72 h, and CCS levels were determined by immunoblotting in whole cell extracts. GAPDH was probed as a loading control. (B), Effects of excess Cu on TEPA-suppressed VEGF expression. HepG2 cells were cultured with TEPA (μM), $CuSO_4$ (μM) alone or in combination at the concentrations indicated for 24 h before incubation under hypoxia for 16 h. VEGF levels in the culture supernatants were determined as described in the Material and Methods. (C), Effects of excess Cu on TEPA-suppressed HIF-1 activation. Cells were transfected with the pH3SVL reporter plasmid containing the luciferase gene under the control of six HIF-1 binding sites from transferrin 3' enhancer, and then treated as described in the Material and Methods. Luicferase activities were measured and normalized to sample protein concentration. (D), Effects of siRNA targeting CCS on HIF-1 activation. Cells were co-transfected with CCS siRNA or mismatched siRNA (mmsiRNA) and the luciferase plasmid before addition of $CuSO_4$ (100 μM) for 24 h. Cells were then subjected to hypoxia for 16 h, and luciferase activities were determined. (Inset of C), efficiency of CCS siRNA on CCS protein levels were determined by immunoblotting. 1, control; 2, transfection reagent; 3, mmsiRNA; 4, CCS siRNA. Data are the mean ± S.D. of three separate culture dishes from one representative experiment. Each experiment was performed three times with consistent results. *$p<0.05$ vs. Controls; $^{\#}$ $p<0.05$ vs.$CuSO_4$ 0 μM and TEPA 50 μM.

Figure 2. **Effects of TEPA on HIF-1α expression and stability**. (A), Western blot analysis for HIF-1α. HepG2 cells were cultured with TEPA (100 μM) for 24 h and then subjected to hypoxia

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for 5 h. Re-oxygenation was performed by exposing the cells in the air for varying times as indicated. HIF-1α protein levels were analyzed by immunoblotting. GAPDH was probed as a loading control. The immunoradiograph is a representative from two separate experiments with consistent results. (B), Immunofluorescent staining of HIF-1α. HepG2 cells were cultured with TEPA (100 μM) or $CuSO_4$ (100 μM) or in combination for 24 h and then subjected to hypoxia for 5 h. a, normoxia; b, hypoxia; c, TEPA treatment followed by hypoxia; d, Cu addition to TEPA-treated cells followed by hypoxia.

Figure 3. **Effect of TEPA on HIF-1 HRE-binding activity**. (A), HepG2 cells were cultured with TEPA (100 μM) for 24 h before exposure to hypoxia for 5 h. Nuclear extracts were prepared and analyzed by EMSA for HRE binding to a ^{32}P-labeled oligonucleotide containing the HIF-1 binding site of the erythropoietin 3' enhancer. The presence of HIF-1 was verified in a competition assay by adding 200-fold molar excess of unlabeled oligonucleotides to the binding reaction prior to addition of labeled probe, and a supershift assay (SS), in which a HIF-1α-specific antibody was added to the binding reaction and incubated for 2 h before resolved, #indicated an overnight incubation with the antibody. Lane 1' normoxia control; lane 2, hypoxia control; lane 3, competition with unlabeled probe; lane 4 and 5, supershift; lane 6, TEPA-hypoxia. C, constitutive binding; NS, non-specific binding. (B) Quantitative analysis of the effect of TEPA on HIF-1 HRE binding was performed from the data obtained from all three independent experiments and expressed as the mean ± SE. * $p<0.05$ vs hypoxia alone. (C) The effect of re-addition of $CuSO_4$ on HIF-1 HRE binding under hypoxic conditions was evaluated by adding 100 μM CuSO4 after the cells were treated with 100 μM TEPA, as described above.

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Figure 4. **Effects of TEPA on p300-binding in HIF-1 transcriptional complex.** (A), HepG2 cells were cultured in the presence or absence of TEPA (100 μM) for 24 h, and then $CuSO_4$ (100 μM) was added to the TEPA-treated cells and cells were then subjected to hypoxia (H) or normoxia (N) for 16 h. Co-immunoprecipitation was performed using a HIF-1α specific antibody in the whole cell lysates. The immunoprecipitants were resolved by SDS-PAGE and p300 was immunoblotted. The lysates were also subjected to Western blot, HIF-1α was probed for loading control. Immunoblot is a representative from three separate experiments with consistent results. (B) Co-immunoprecipitation of HIF-1α by anti-p300. . N, normoxia control; H, hypoxia control; TEPA-H, hypoxia after TEPA treatment; TEPA-Cu-H, hypoxia after addition of $CuSO_4$ (100 μM) to the TEPA treated cells.

Figure 5. **Effects of siRNA targeting FIH-1 on HIF-1 transcriptional activity**. HepG2 cells were co-transfected with the luciferase reporter plasmid and FIH-1siRNA. Cells were then incubated with TEPA (100 μM) for 24 h and then subjected to hypoxia for 16 h. (A) Western blotting of FIH-1 protein levels. Left panel, the effect of TEPA on FIH-1 protein level; right panel, efficiency of FIH-1 gene silencing by siRNA. (B) Luciferase activity. Data are the mean ± S.D. of three separate culture dishes from one representative experiment, which was performed two times with consistent results. *p<0.05 vs. control. (C) Effect of FIH-1 siRNA on HRE binding. Procedures were as the same as described in Fig. 3.

Figure 6. **Effects of TEPA on DFO-induced VEGF expression and HIF-1 activation**. HepG2 cells were cultured with TEPA (100 μM) for 24 h before addition of DFO (100 μM) for 16 h. VEGF levels in the culture supernatants (A) and luciferase activities (B) were determined. Data are

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mean ± S.D. of three separate culture dishes from one representative experiment that was performed two times with consistent results. *p<0.05 vs controls.

Figure 7. Graphical representation of the proposed mechanisms of effects of Cu chelation by TEPA on HIF-1 transactivity. Upon stabilized by hypoxia or other stimuli, HIF-1α binds HIF-1β and translocates into nucleus. The heterodimer then binds to HRE sequence and co-factor p300, and starts transcription of target genes. Cu chelation by TEPA would activate FIH-1 resulting in a hydroxylation of HIF-1α and inhibition of its binding to p300. It would also attenuate the binding of HIF-1α to its target gene sequences leading to suppression of its transcriptional activity. CCS is a Cu chaperon and its deletion mimics the same effect as Cu chelation by TEPA.

Fig 1

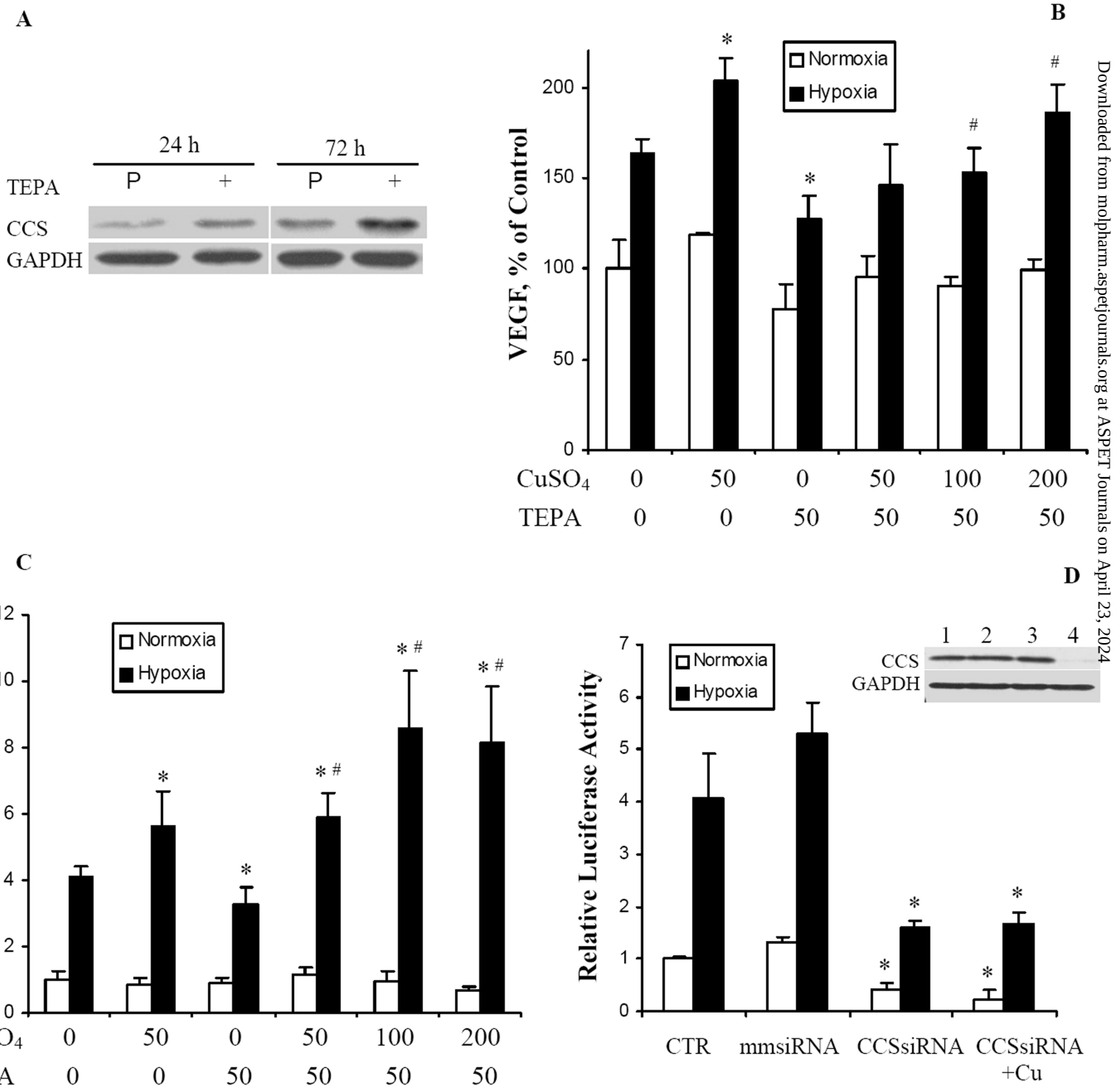


Fig 2

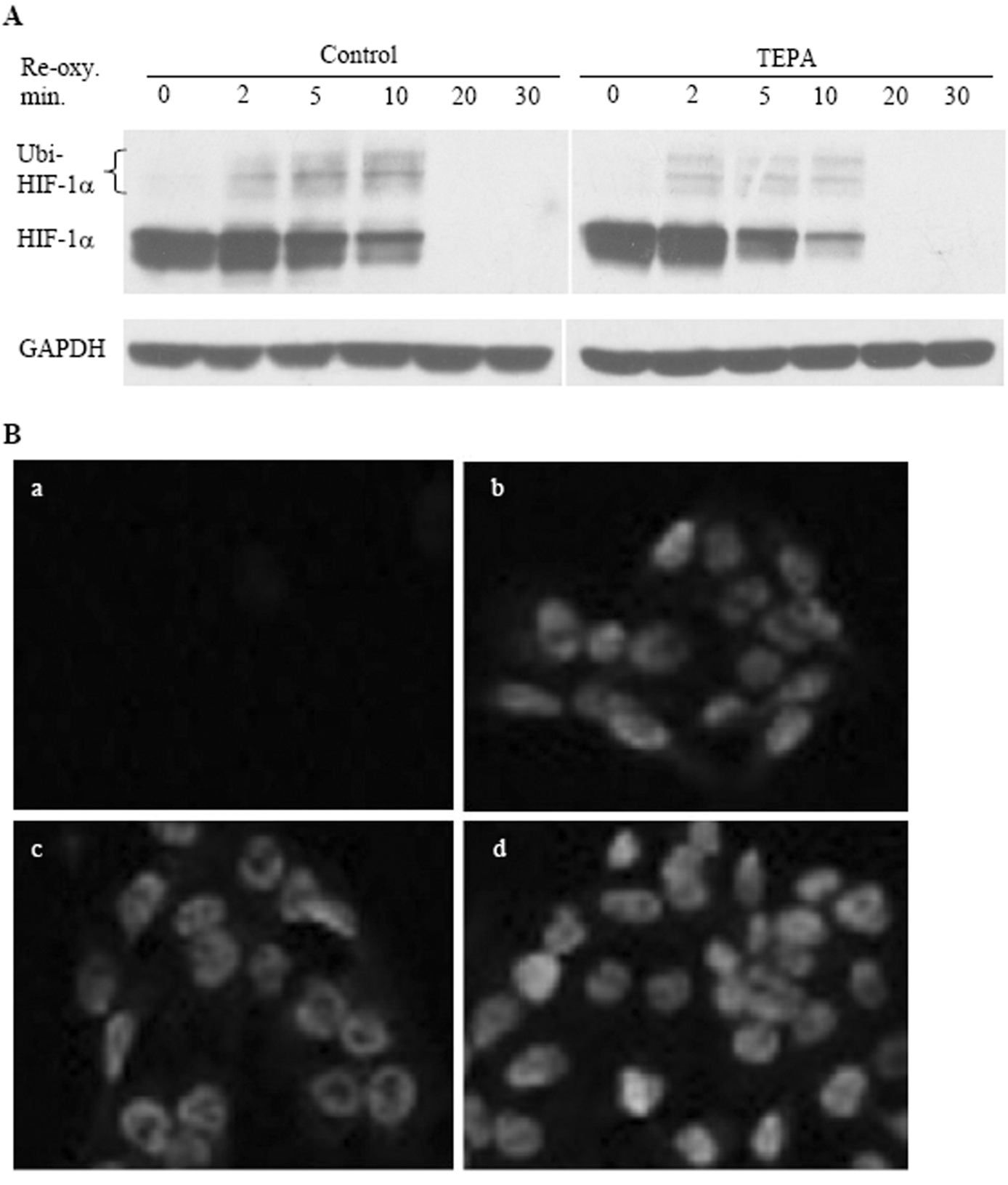
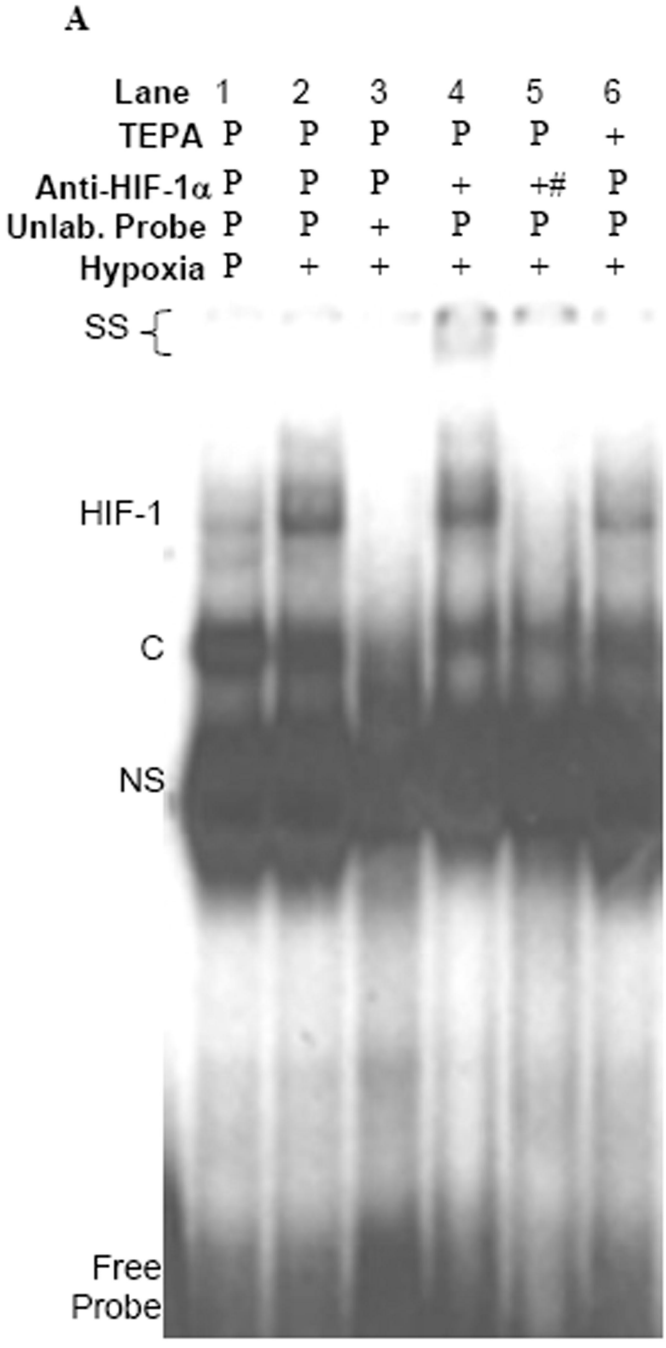
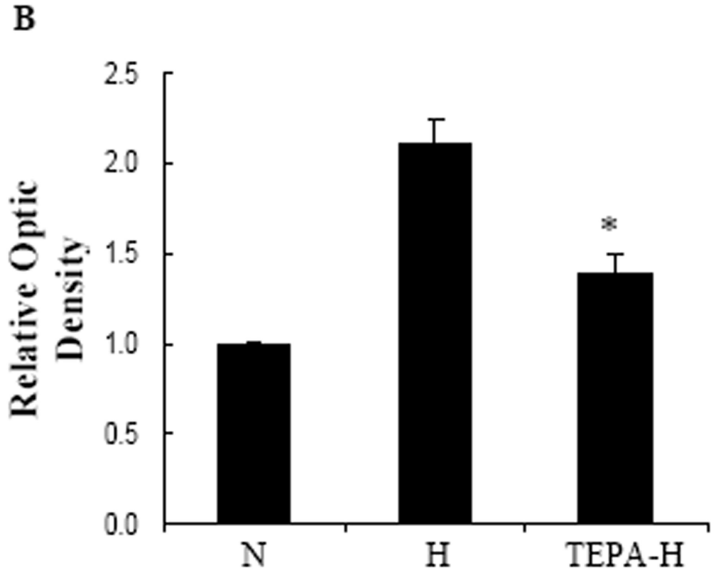


Fig 3





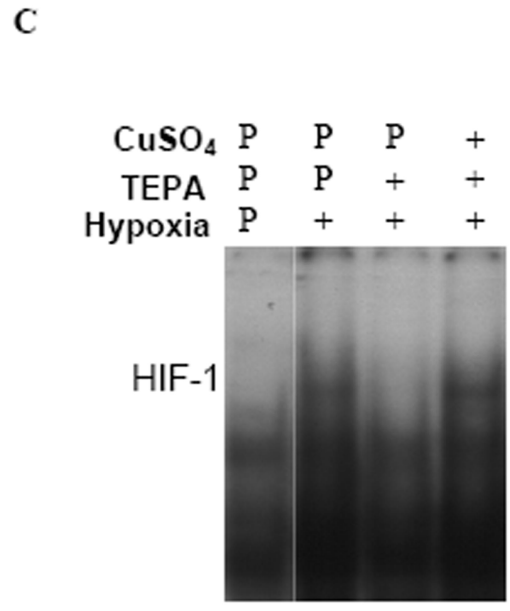


Fig 4

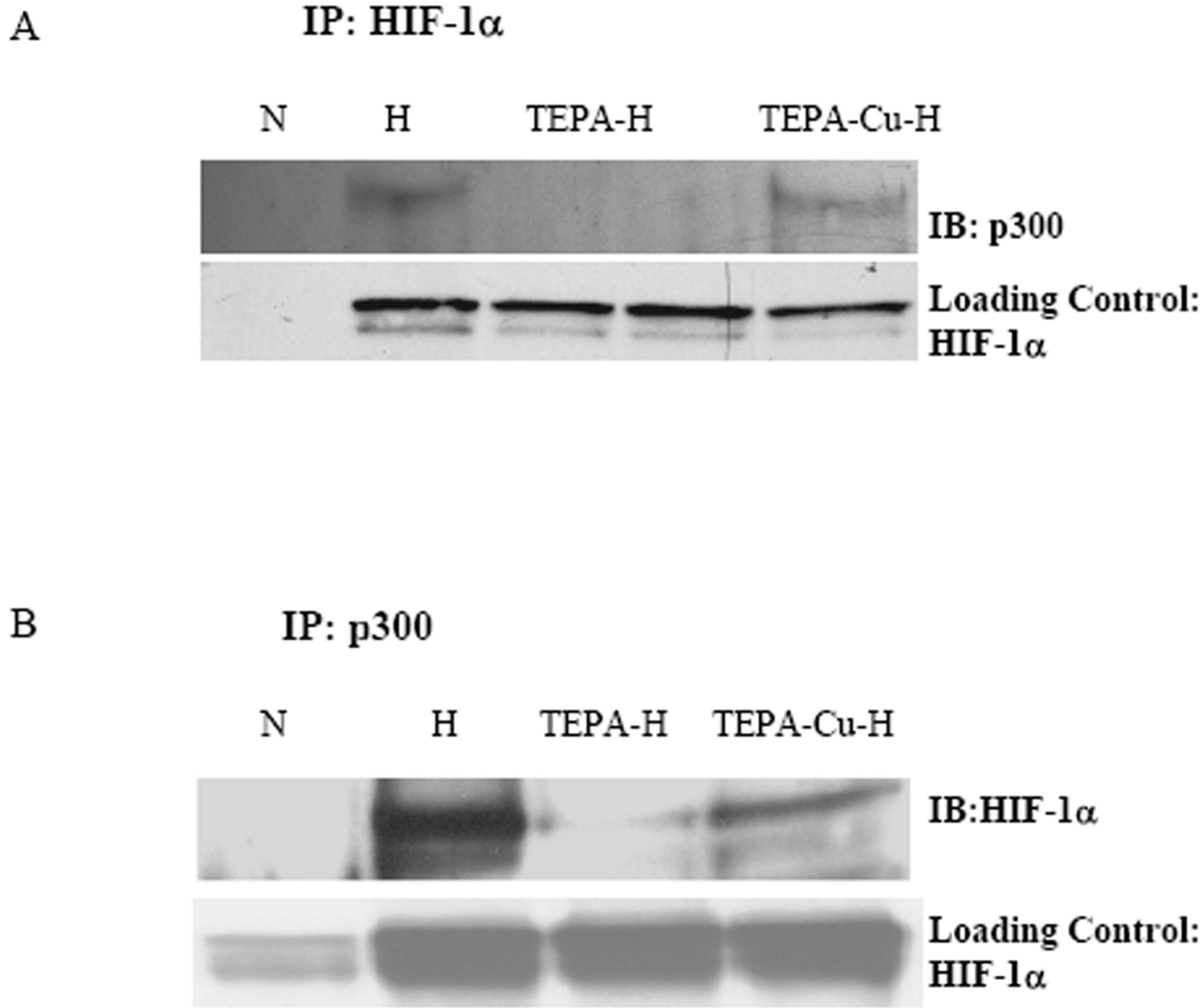
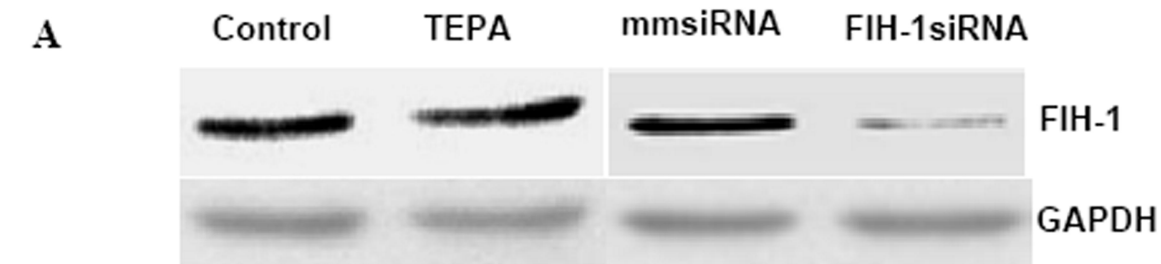
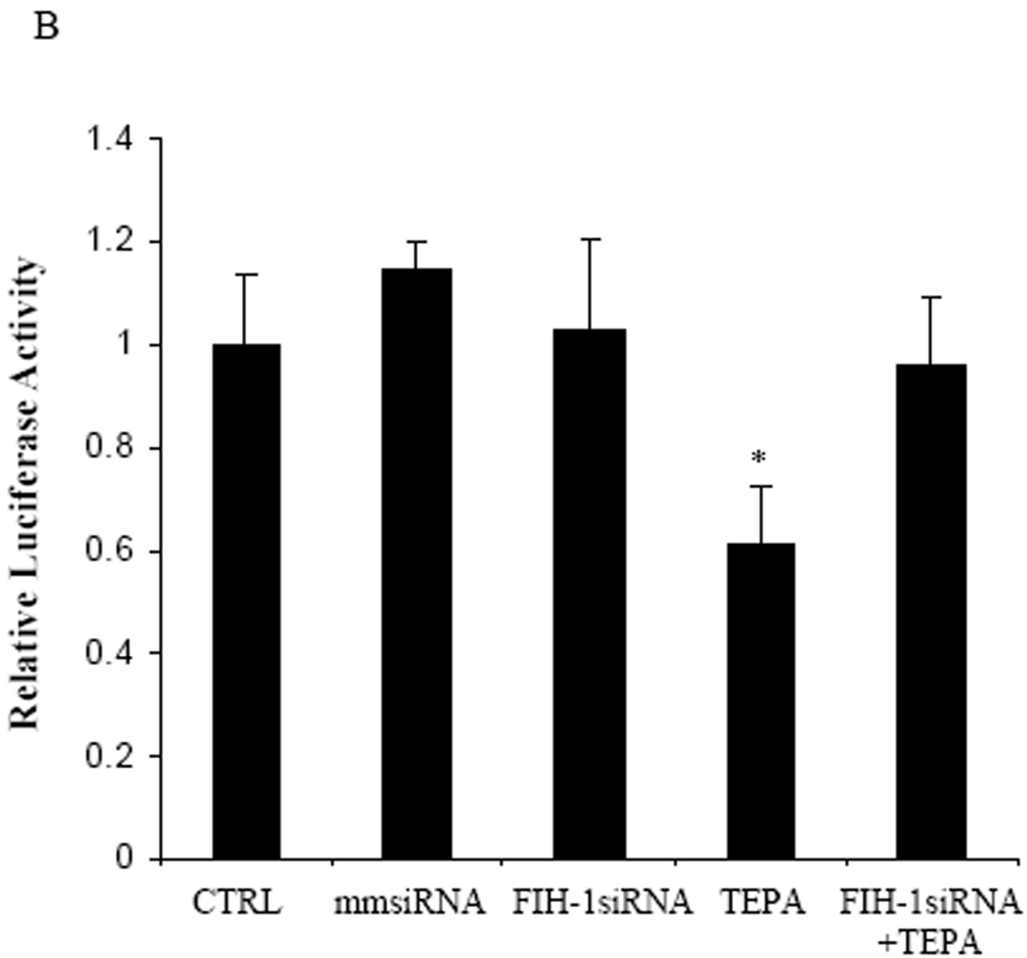


Fig 5





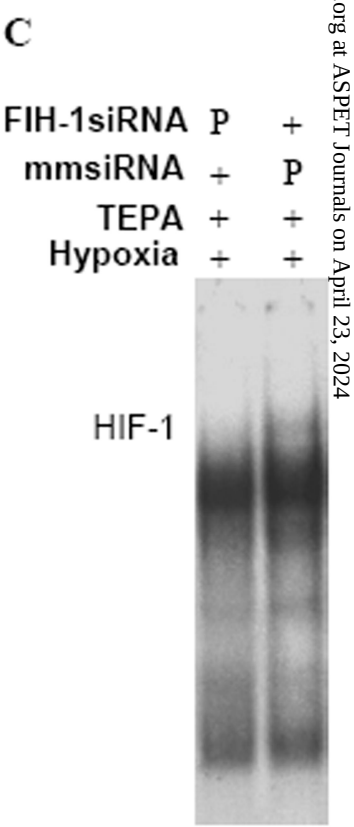
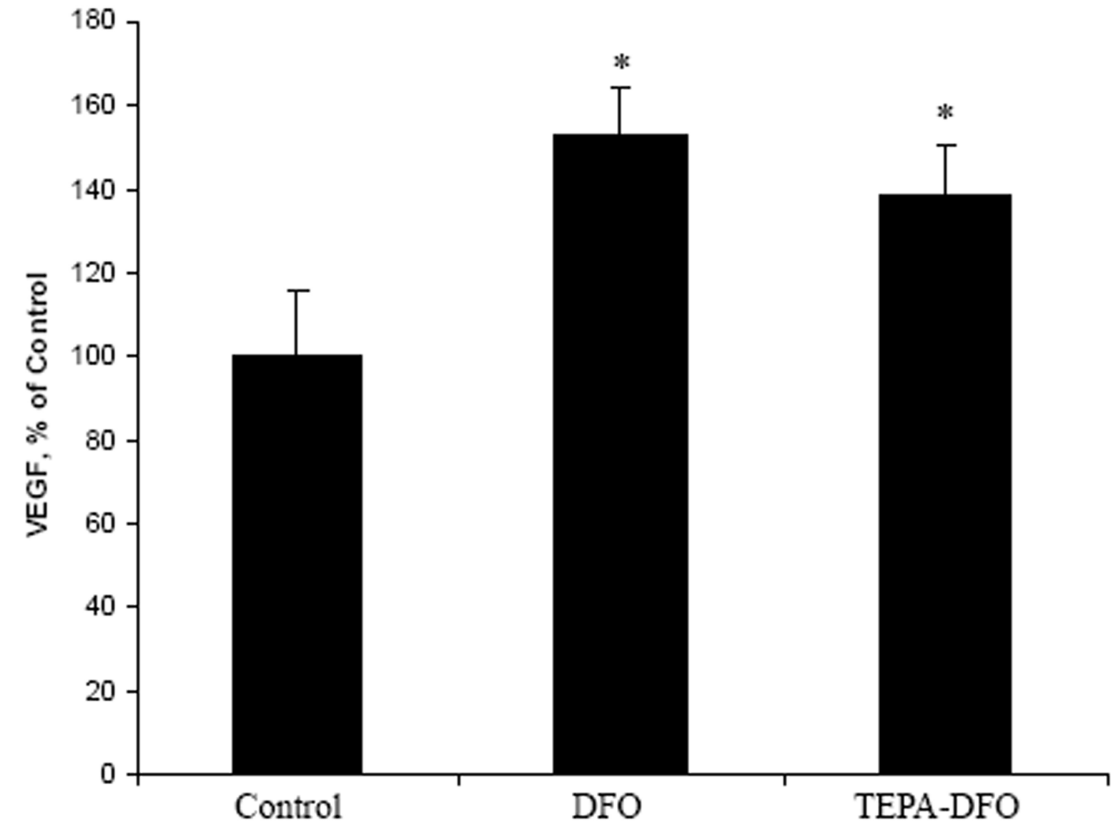


Fig 6



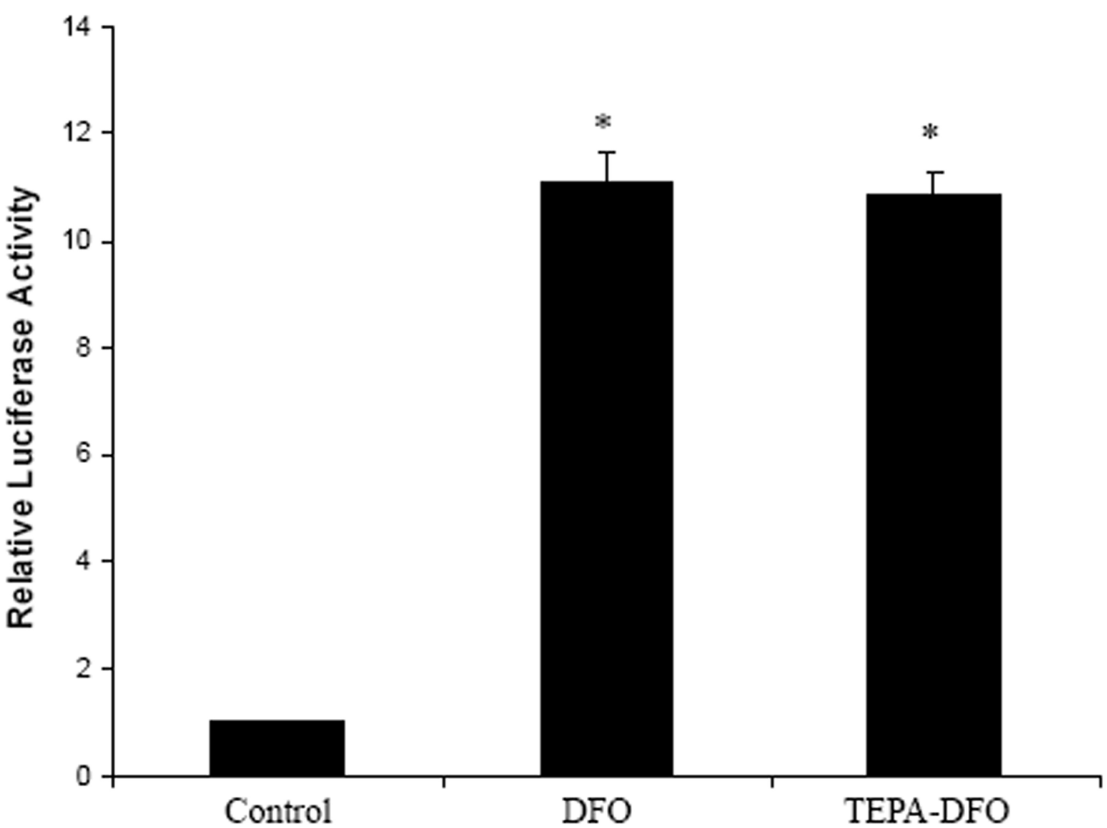


Fig 7