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Hypoxia inducible factor-1 (HIF-1)-mediated upregulation of vascular endothelial growth factor (VEGF) has been implicated in angiogenesis associated with malignancies. HIF-1 consists of a constitutively expressed HIF-1β subunit, and a hypoxia-inducible HIF-1α subunit. Hypoxic induction of HIF-1α correlates with increased transcriptional activation of its downstream target genes, including VEGF. Epidemiologic and laboratory studies indicate that green tea has cancer preventive activity which has been attributed to its polyphenol components, the major one being epigallocatechin gallate (EGCG). This study investigated the effect of EGCG on normoxic VEGF expression in PC-3ML human prostate cancer cells. In contrast to previous studies where EGCG inhibited VEGF expression in breast and colon cancer cell lines, our results demonstrated that EGCG has the ability to upregulate HIF-1α transcription factor via inhibition of prolyl hydroxylation and subsequent von Hippel-Lindau protein interaction. HIF-1α upregulation by EGCG led to increased VEGF promoter activity and protein expression.

EGCG AND ITS ROLE IN PROSTATE CANCER ANGIOGENESIS

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EGCG AND ITS ROLE IN PROSTATE CANCER ANGIOGENESIS

THESIS

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LIST OF ABBREVIATIONS

EGCG	Epigallocatechin gallate
ODD	Oxygen-dependent degradation domain
HIF-1	Hypoxia inducible factor-1
HPH	HIF-1 prolyl hydroxylase
pVHL	von Hippel Lindau protein
HRE	Hypoxia response element
VEGF	Vascular endothelial growth factor
PCa	Prostate cancer
DFX	Desferrioxamine
AP-1	Activator protein-1

CHAPTER I

INTRODUCTION

During hypoxia, hypoxia inducible factor-1 (HIF-1) activates transcription of a vast array of hypoxia-responsive genes including those involved in angiogenesis, erythropoiesis, glucose metabolism, cell survival and tissue invasion (1). HIF-1, an αβ heterodimeric transcription factor, consists of a constitutively expressed HIF-1β subunit, and a hypoxia-inducible HIF-1α subunit. Under normoxic conditions, HIF-1α is hydroxylated at proline residues 402 and 564 within an oxygen-dependent degradation domain (ODD) (2-5) by Fe^{2+}-dependent HIF-1 prolyl hydroxylases (HPH) that use O_2 as a substrate (2, 6). Because O_2 appears to be rate limiting for prolyl hydroxylase activity, these enzymes may represent cellular O_2 sensors (2,7,8). The von Hippel-Lindau protein (pVHL), the substrate recognizing component of an E3 ubiquitin ligase complex, links hydroxylated HIF-1α to the ubiquitination machinery and proteasome-mediated degradation (9-11). This mechanism ensures very low cellular HIF-1α levels under normoxia. Under hypoxic conditions, HPH activity is inhibited due to lack of O_2 thereby allowing accumulation of HIF-1α which then translocates to the nucleus and associates with HIF-1β subunit (12). The HIF-1αβ heterodimer binds to hypoxia response elements (HRE) (5'-CGTG-3') found in the promoter/enhancer regions of HIF-1 target genes, thereby activating their expression (8). A diagrammatic representation of the HIF-1α pathway can be found in diagram 1.

Vascular endothelial growth factor (VEGF), one of the most potent inducers of angiogenesis, has been known to stimulate the proliferation and migration of vascular endothelial cells (13-15). VEGF is also one of the HIF-1 target genes whose expression is upregulated in normal and cancer cells in response to hypoxia. HIF-1-mediated upregulation of VEGF has been implicated in angiogenesis associated with malignancies (16-18). It has been determined that human prostate cancer (PCa) stained positive for VEGF by immunohistochemical studies and that a PCa cell line, DU-145, expressed VEGF upon cytokine stimulation (19). Similarly, the highly metastatic LNCaP-LN3 cell line overexpressed VEGF when compared with the parental LNCaP cell line (20). A significant correlation has been reported between increasing hypoxia in prostate tumors and the percentage of cells staining positive for VEGF (21). Thus, the angiogenic and metastatic phenotype of PCa seems to be directly correlated with increased expression of VEGF as well as other angiogenic factors (22).

Epidemiological studies have shown that the Japanese and Chinese population, which traditionally consume a diet having low fat and high fiber contents and several cups of tea, especially green tea, have one of the lowest rates of PCa in the world (44). Epigallocatechin gallate (EGCG) is the major epicatechin component in green tea leaves, and has recently been studied intensively as an anticarcinogenic and antiangiogenic agent (23,24). Several groups have shown the cancer-preventive effects of tea polyphenols on various cancers including skin, lung, pancreas, breast, and prostate (25). Cao and Cao (24) have shown that EGCG suppresses bovine capillary endothelial cell growth *in vitro*, and also the formation of new blood vessels in chick chorioallantoic membrane. EGCG treatment of androgen-sensitive LNCaP and androgen-insensitive DU-145 resulted in G0/G1 cell cycle arrest and induction of apoptosis (40-50% of cells) in a dose- and time-dependent manner (40-80 μg/ml and 48 hr) (26). In animal studies, oral feeding of a polyphenolic fraction isolated from green tea (GTP) at 0.1% to transgenic

adenocarcinoma of mouse prostate (TRAMP) mice significantly inhibits PCa development and increases survival in these mice (27), and also lowers VEGF expression (28).

EGCG has also been shown to inhibit the induction of VEGF in human cancer cells (29,30). In HT29 human colon carcinoma cells, EGCG (10-100 μg/ml) inhibited Erk-1 and Erk-2 phosphorylation and the increase in VEGF expression induced by serum starvation (30). In MDA-MB231 breast cancer cells and human umbilical vein endothelial cells, EGCG (40 μg/ml) significantly decreased VEGF secretion into conditioned media (29). Recently, Nam *et al.* reported that EGCG (10-40 μM = 4.6-18.4 μg/ml) inhibited proteasomal activity in intact Jurkat leukemic cells and prostate cancer cells (PC-3 and LNCaP) (31). In addition, most flavonoids possess metal chelating property due to the presence of a catechol structure (25). They can bind and thus decrease the level of cellular ferric and ferrous ions. Thus, these data imply a potential regulation of HIF-1α protein by EGCG since HIF-1α protein is hydroxylated by Fe^{2+}-dependent HPH and rapidly degraded by proteasomes under normoxia (32).

This study investigated the effect of EGCG on VEGF expression and on the regulation of HIF-1α transcription factor in normoxic prostate cancer cells. Herein, we report that EGCG increases VEGF expression, in part mediated by increasing HIF-1α proteins in prostate cancer cells in normoxia.

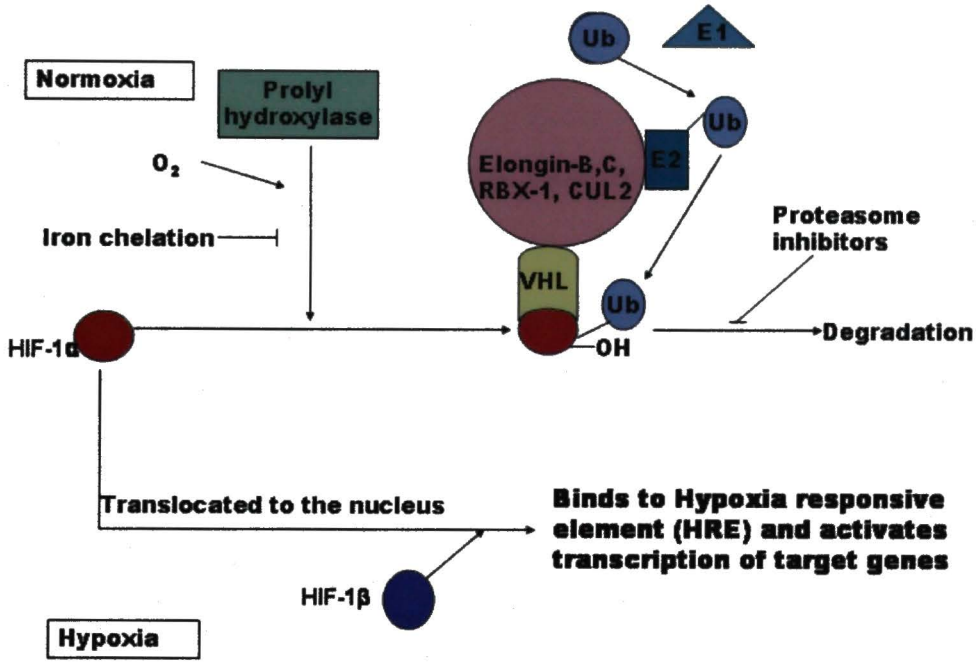

Diagram 1: HIF-1α pathway. Under normoxic conditions, HIF-1α is hydroxylated at Pro ^{402} and Pro^{564} within the HIF-1α ODD by Fe^{2+}-dependent HIF-1 prolyl hydroxylase thereby allowing for HIF-1α recognition by an E3 ubiquitin ligase complex. pVHL, the substrate recognizing component of the E3 ubiquitin ligase complex, links the hydroxylated HIF-1α to the ubiquitination machinery and proteasome-mediated degradation. Under hypoxic conditions, HIF-1 prolyl hydroxylase is inhibited due to the lack of O_2, hence, HIF-1α accumulates and translocates to the nucleus where it associates with the HIF-1β subunit. The HIF-1αβ heterodimer binds to hypoxia response elements found in the promoter/enhancer region of HIF-1 target genes, and in association with other transcriptional co-activators activates the expression of these genes.

CHAPTER II

MATERIALS AND METHODS

Reagents- (-)-Epigallocatechin gallate (EGCG), $CoCl_2$, ferrous sulfate ($FeSO_4$), desferrioxamine (DFX), Protein G-agarose beads and protease inhibitor cocktail were obtained from Sigma. 2-oxoglutarate was obtained from Fisher Scientific. Primary antibodies to ubiquitin, HIF-1α and HIF-1β were from Santa Cruz Biotechnology, and anti-β-actin and anti-FLAG antibodies were from Sigma. Secondary antibodies, horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG, M-PER mammalian protein extraction reagent and neutravidin beads were from Pierce. Dual Luciferase reporter assay system, BL21 (DE3) pLysS competent cells, isopropyl-β-D-thiogalactopyranoside (IPTG), MagneHis protein purification system, TNT T7 Coupled Reticulocyte Lysate System and RNase inhibitor were from Promega. PD-10 columns and L-[^{35}S] methionine were from Amersham Biosciences. Biotinylated test and positive control peptides were synthesized by Alpha Diagnostic International. Their sequences correspond to the Oxygen-dependent Degradation Domain (ODD) of HIF-1α and are as follows: biotin-Acp-DLDLEMLAP*YIPMDDDFQL-COOH. P* represents Pro^{564}, the hydroxylation site for HPH, and this amino acid residue is hydroxylated in the positive control peptide.

Plasmid constructs- pGL3-6xHRE-Luc was a generous gift from Dr. Peter RatCliffe (University of Oxford) (32), and contained six copies of hypoxia response element (HRE) from the erythropoietin gene promoter linked to the thymidine kinase basal promoter and firefly luciferase

gene. pGL3-hVEGF-Luc was a generous gift from Dr. Richard P. DiAugustine (National Institute of Environmental Health Sciences, Research Triangle Park, NC) and contained the human VEGF promoter linked to the firefly luciferase reporter gene (33). pRL-TK (Promega) was used as transfection efficiency control and contained the thymidine kinase promoter linked to Renilla luciferase gene. pVHL in pcDNA3.1-V5 His and HPH-2C in pHIS-parallell vector were generous gifts from Dr. Richard K. Bruick (University of Texas Southwestern Medical Center, Dallas, TX). pCEP-HIF-1α was from ATCC, and pFLAG-CMV3 from Sigma. pFLAG-HIF-1α expression vector coding for FLAG-epitope-tagged HIF-1α protein was constructed by inserting the HIF-1α cDNA coding sequence into the HindIII/BamHI site of pFLAG-CMV3 (Sigma).

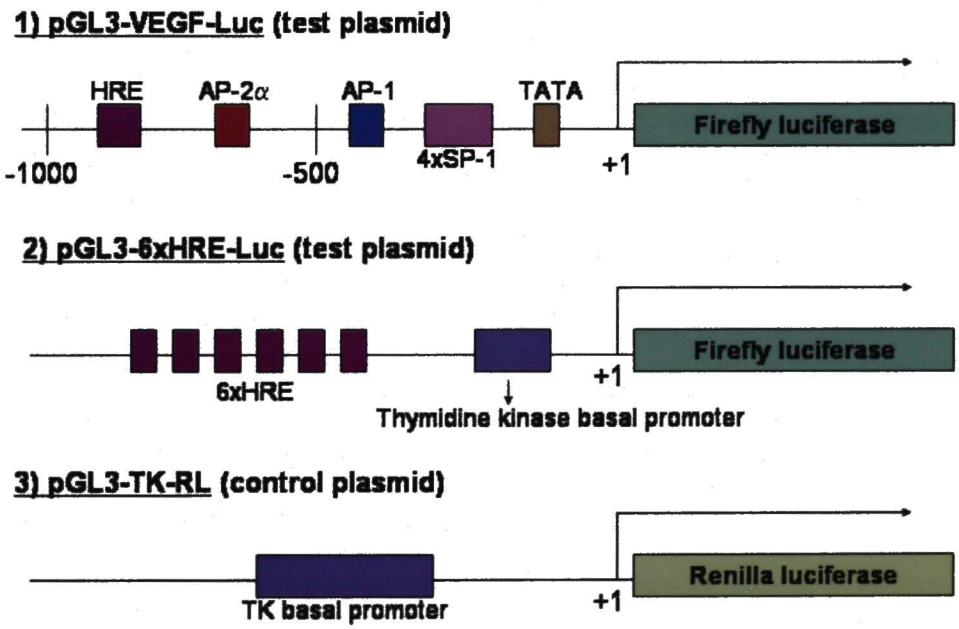

Diagram 2: Plasmid constructs used in the dual luciferase reporter assay. *The relative positions of the transcription factor binding sites are not to scale.

Cell culture- The PC-3ML prostate carcinoma cell line, a subline of the PC-3 cell line, was a generous gift from Dr. Mark Stearns (MCP-Hahnmann University, Philadelphia) (34). It has been characterized as a cell line with a highly invasive and bone-targeting metastatic phenotype (34). PC-3ML cells were maintained in F-12K Nutrient Mixture (Kaighn's Modification) (Invitrogen/Gibco) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin sulfate and 100 units/ml penicillin G sodium in a humidified 5% CO_2 incubator at 37°C, and routinely passaged when 80% to 90% confluent.

Transient transfections and reporter gene assay- PC-3ML cells were grown in 48-well plates in F-12K medium without antibiotics until 90% confluent. PC-3ML cells were then transiently transfected with pGL3-hVEGF-Luc or pGL3-6xHRE-Luc as test plasmids along with pRL-TK using Lipofectamine 2000 (Invitrogen). After 4 h post-transfection, medium was replaced with fresh F-12K serum free medium with or without EGCG. PC-3ML cells were harvested 24 h later for dual luciferase assays (Promega) to determine VEGF or HRE-mediated transcriptional activity. The firefly luciferase expression from pGL3-hVEGF-Luc or pGL3-6xHRE-Luc and Renilla luciferase from pRL-TK were measured sequentially from a single sample, in a TD-20/20 Luminometer (Turner Designs) according to the Dual-Luciferase Reporter System protocol (Promega). The activity of the pGL3-hVEGF-Luc or pGL3-6xHRE-Luc was normalized to the activity of the pRL-TK internal control to minimize experimental variability caused by differences in cell viability or transfection efficiency, and then represented as relative luciferase activity on a bar graph.

Whole cell lysate preparation- PC-3ML cells were harvested by scraping and washed in ice-cold PBS and lysed in M-PER reagent (Pierce Chemical, Rockford, IL) supplemented with

protease inhibitors (Sigma) at 100 µl/10^6 cells. After 15 min incubation on ice, lysates were cleared by centrifugation at 10,000 rpm, at 4°C. The resulting lysates were stored at –80°C until needed. Protein concentration was measured by bicinchoninic acid (BCA) assay (Sigma).

Immunoblot analysis: Whole cell lysates (25-50 µg of protein) were separated on a 4-20% gradient SDS polyacrylamide gel (Cambrex) under reducing conditions and then transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature in T-PBS, pH 7.5 (PBS with 0.2% Tween-20) with 5% non-fat dry milk. After washing, the blot was incubated with primary antibody for 1 h at RT. The blot was washed 3 times in T-PBS and incubated with HRP-coupled secondary antibody (Pierce Chemical, Rockford, IL) for 1 h at RT. After extensive washing, the bands were detected using SuperSignal West Femto Maximum Sensitivity Substrate System (Pierce). The resulting chemiluminescence was visualized by ChemiImager™ 4400 equipped with a ChemiNova™ CCD camera (Alpha Innotech Corporation, San Leandro, CA). Densitometric analysis of protein bands was performed using AlphaEase™ Software (Alpha Innotech Corporation). Biotinylated protein standard (Bio-Rad) and streptavidin-HRP conjugates were used to estimate protein MW on the blot. After stripping, the blot was reprobed with β-actin antibody to determine the equivalence of protein loading. HIF-1α expression was normalized to β-actin density from the same sample and expressed as a fold-change, with HIF-1α in control PC-3ML cells set at 1.

VEGF ELISA- PC-3ML cells were seeded in 48-well plates at 40,000 cells/well and 3 wells per treatment. After 24 h, the medium was replaced with fresh serum-free F12K medium. Cells were then treated with EGCG at 0, 10, 20, or 40 µg/ml for 24 h. Culture supernatants were harvested for VEGF ELISA using VEGF ELISA kit essentially as described in the protocol provided by the

manufacturer (R&D Systems). Briefly, PC-3ML culture supernatants were added to wells of VEGF microplate coated with a monoclonal antibody against VEGF and incubated for 2 h at room temperature. The plate was washed 3 times with wash buffer (provided in the kit). VEGF conjugate was added, and the plate was incubated for 2 h at room temperature. The plate was washed and color reagent was added. Color development was stopped by the addition of stop solution and the absorbance was measured in an Elx808IU Ultra Microplate Reader (BIO-TEK Instruments, Inc.) at a wavelength of 450 nm and reference wavelength of 630 nm. The VEGF concentration in each well was calculated using the equation of standard curve and normalized according to the cell number in each well and expressed as picogram per 10^6 cells. Cell number was determined by WST assay (Roche) according to the manufacturer's instruction.

Ubiquitination assay- PC-3ML cells were transiently transfected with pFLAG-HIF-1α plasmid and 4 h later, treated with 20 μg/ml EGCG, 300 μM $FeSO_4$ or 20 μg/ml EGCG + 300 μM $FeSO_4$ in serum-free F-12K medium. After 24 h, the cells were collected and lysed in M-PER mammalian protein extraction reagent (Pierce) supplemented with protease inhibitors. FLAG-HIF-1α protein was immunoprecipitated from the cell lysates with FLAG antibodies conjugated to protein G-agarose beads. The samples were boiled in 2x SDS sample buffer, and the beads were removed by centrifugation. Proteins were electrophoretically separated on 4-20% gradient gels, followed by immunoblot analysis with antibodies to ubiquitin or HIF-1α.

In-vitro HPH activity assay- His-tagged HPH-2C (His-HPH-2C) was expressed by transforming BL21 (DE3) pLysS cells with HPH-2 cDNA in pHIS-parallell vector. Cells expressing His-HPH-2C were induced with 1 mM IPTG overnight at 20°C. The induced cells were lysed, and His-HPH-2C was purified using the MagneHis protein purification system (Promega) according to the

protocol provided by the manufacturer. ^{35}S-labeled pVHL (^{35}S pVHL) was produced by carrying out an in-vitro transcription translation (IVTT) reaction with TNT T7 Coupled Reticulocyte Lysate System (Promega) using pcDNA3.1-V5His-VHL plasmid in the presence of L-[^{35}S]-methionine according to the manufacturer's instructions. The IVTT reaction (supplemented with protease inhibitor cocktail) was desalted using PD-10 desalting columns (Amersham Biosciences). Briefly, the PD10 column was equilibrated with 25 ml ice-cold buffer (20 mM Tris-Cl pH 7.5, 100 mM NaCl, 1 mM EDTA). The IVTT reaction was then loaded onto the column and eluted with 15 ml of the same buffer. 0.5 ml fractions were collected and scintillation counted and the peak pVHL fraction was used in the HPH activity assay. For the HPH activity assay, 20 µl neutravidin beads were incubated with 10 µg biotinylated HIF-1α ODD (test peptide) or biotinylated-Pro^{564}hydroxylated HIF-1α ODD (positive control peptide) in 25 mM Tris-Cl pH 7.5 at room temperature for 1 h. Beads were washed 3 times with 1x Reaction buffer (10 mM Tris-Cl pH 7.5, 2.5 mM KCl, 0.75 mM $MgCl_2$, 1 mM ascorbate, 1 mM 2-oxoglutarate, 0.5 mM DTT) to remove unbound peptides. To each reaction containing 20 µl peptide-bound beads, 25 µl 2x Reaction buffer containing 10 µM Fe^{2+}, EGCG (10-40 µg/ml) and 0.5 µg His-HPH-2C were added and incubated at room temperature for 1 h. The beads were washed 3 times with NETN buffer (20 mM Tris-Cl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40) and then incubated in 500 µl EBC buffer (50 mM Tris-Cl pH 8.0, 120 mM NaCl, 0.5% NP-40), 5 µl ^{35}S pVHL, and EGCG (10-40 µg/ml) for 20 min on ice. The beads were washed 3 times with NETN buffer, and the radioactivity of the ^{35}S pVHL bound to the biotinylated peptides was quantified by liquid scintillation counting in a Beckman 6000IC liquid scintillation counter. Diagram 3 illustrates the key steps involved in the *in-vitro* HPH activity assay.

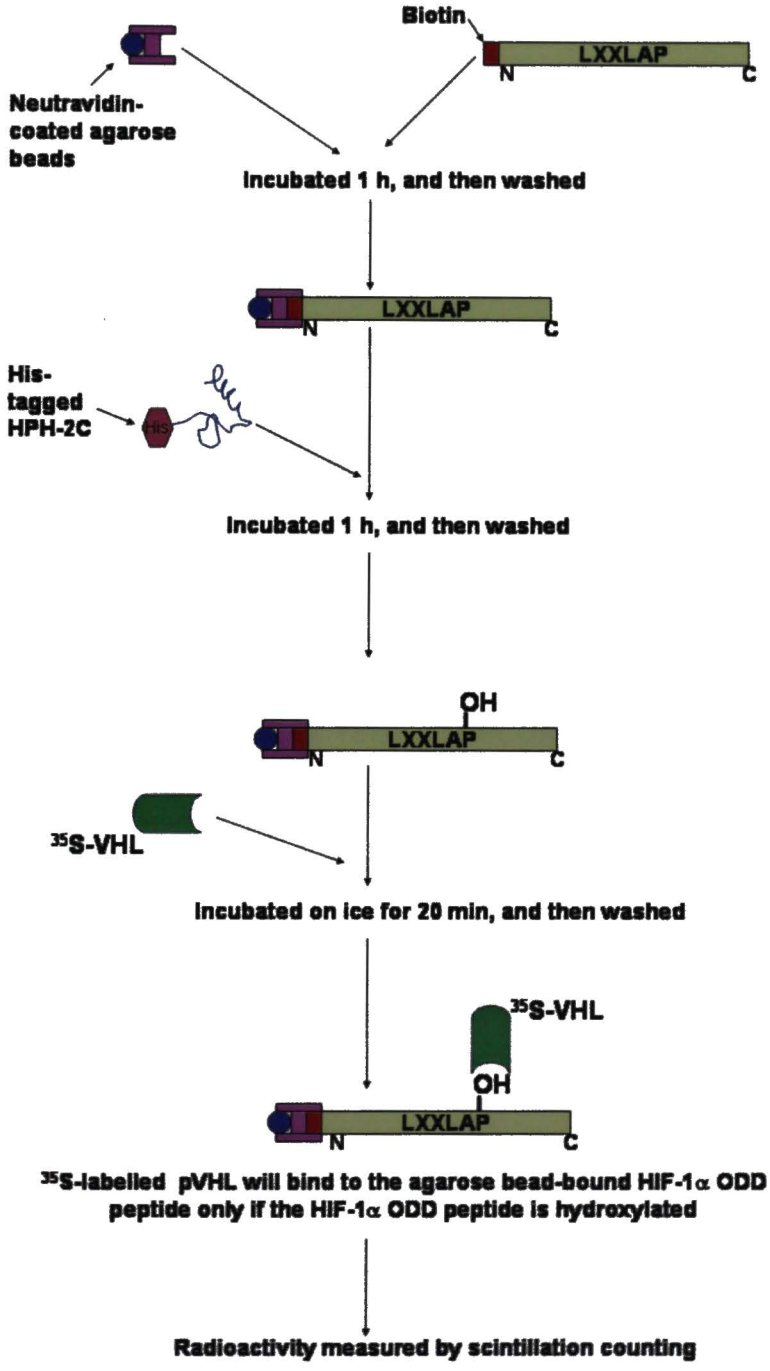

Diagram 3: In-vitro HPH activity assay

CHAPTER III

RESULTS

EGCG increases VEGF expression under normoxia- Several studies have reported that EGCG inhibited the expression of VEGF in breast cancer cells and colon cancer cells in *in-vitro* studies (29,30). A five-fold higher expression of VEGF has been reported in the dorsolateral prostate of TRAMP mice compared with littermate nontransgenic mice (28). In addition, oral feeding of 0.1% GTP inhibited VEGF expression in TRAMP mice (28). EGCG's *in vitro* effect on prostate cancer cells was examined in order to characterize the molecular mechanism of action of EGCG on VEGF expression in prostate cancer. PC-3ML cells are a highly metastatic variant of PC-3 cells, and have been shown to express VEGF (35). PC-3ML cells were transiently co-transfected with pGL3-hVEGF-Luc as a test plasmid along with pRL-TK as a transfection efficiency control, and then treated with EGCG at 0, 10, 20, or 40 μg/ml for 24 h. Surprisingly, EGCG treatment ($\geq$ 20 μg/ml) increased VEGF promoter activity by about 2.5-fold in PC-3ML cells compared to that of control cells (Fig. 1A). Next, VEGF protein secretion from EGCG-treated PC-3ML cells was examined by ELISA. As shown in Fig. 1B, EGCG treatment increased VEGF protein secretion from PC-3ML cells up to 3-fold in a dose-dependent manner.

EGCG upregulates HRE-mediated transcription under normoxia- The HIF-1 transcription factor has been shown to be important in VEGF transcription in prostate cancer cells (1,16,33). To determine the effect of EGCG on HIF-1 transcription factor, a reporter gene assay was used in

which PC-3ML cells were transiently co-transfected with pGL3-6xHRE-Luc to test HIF-1 transcriptional activity, and pRL-TK as a transfection efficiency control. PC-3ML cells were treated with increasing amounts of EGCG for 24 h, followed by luciferase assay. As shown in Figure 2, HRE-mediated transcription was increased up to 7-fold by EGCG treatment (at 40 μg/ml) in a dose dependent manner.

EGCG increases HIF-1α protein levels- In a recent study, EGCG has been shown to inhibit proteasome activity in PC-3 cells (31). Hence, we hypothesized that EGCG might prevent the degradation of HIF-1α protein, leading to the accumulation of HIF-1α protein and increased HIF-1 transcriptional activity. To test this hypothesis, we examined HIF-1α protein regulation following EGCG treatment. PC-3ML cells were treated with 20 μg/ml EGCG or 100 μM $CoCl_2$, a hypoxia mimic, under serum-free conditions. When compared to the untreated control, a 3-fold increase in HIF-1α protein levels was seen in PC-3ML cells following a 24-h treatment with 20 μg/ml EGCG (Fig. 3). The hypoxia mimic, $CoCl_2$, which has been known to induce HIF-1α protein expression by blocking its degradation (36) modestly increased HIF-1α protein level. However, EGCG did not significantly affect HIF-1β expression (Fig. 3). In dose response experiments, EGCG increased HIF-1α protein levels in a dose-dependent manner (Fig. 4).

EGCG attenuates ubiquitination of HIF-1α- If EGCG increased HIF-1α protein by inhibiting proteasomal degradation (31), the ubiquitinated form of HIF-1α protein would accumulate in EGCG-treated cells. To test this hypothesis, HIF-1α ubiquitination with or without EGCG treatment was determined. PC-3ML cells were transiently transfected with pFLAG-HIF-1α expression vector coding for FLAG-epitope-tagged HIF-1α protein. FLAG-HIF-1α protein was

immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-ubiquitin antibody to detect ubiquitination of HIF-1α. However, immunoblotting revealed no significant accumulation of ubiquitinated HIF-1α protein in the EGCG-treated cells (Fig. 5, lane 2) when compared to the control cells (Fig. 5, lane 1). Instead, we observed a decrease in ubiquitinated HIF-1α in EGCG-treated cells. $FeSO_4$ alone also seemed to decrease ubiquitinated HIF-1α levels. However, addition of exogenous $FeSO_4$ together with EGCG restored levels of ubiquitinated HIF-1α protein to that of control cells. The blot was also probed with anti-HIF-1α antibody to confirm immunoprecipitation of HIF-1α protein (Fig. 5). Taken together, these data suggest that EGCG acted prior to the ubiquitination step to prevent HIF-1α degradation which required Fe^{2+}.

Fe^{2+} abolishes EGCG-induced increase in HRE-mediated transcription and VEGF expression –The effect of exogenous Fe^{2+} on the EGCG-mediated increase in promoter activity of pGL3-6xHRE-Luc and VEGF protein secretion in PC-3ML cells was tested. PC-3ML cells were transiently co-transfected with pGL3-6xHRE-Luc and pRL-TK plasmids and treated with increasing concentration of EGCG in the absence or presence of 300 μM $FeSO_4$ (Fig. 6). The luciferase activity from pGL3-6xHRE-Luc in EGCG-treated cells (40 μg/ml) was increased 2.7 fold compared to that of control cells. However, the EGCG-mediated increase in HRE-promoter activity was abolished by the addition of exogenous $FeSO_4$. $FeSO_4$ by itself had no significant effect on HRE-promoter activity. Desferrioxamine (DFX), a known iron chelator, increased HRE-mediated transcription by 4.4 fold, and this increase was abolished by the addition of $FeSO_4$. Moreover, the addition of $FeSO_4$ abolished EGCG-mediated increase in VEGF protein secretion from PC-3ML cells (Fig. 7). Thus, these results strongly suggest that the metal ion chelating activity of EGCG played an important role in increasing HRE-mediated VEGF expression.

EGCG inhibits HPH activity- HPH requires Fe^{2+} for its activity to hydroxylate HIF-1α. Hence it was hypothesized that EGCG inhibits HPH activity and consequently inhibits HIF-1α ubiquitination and degradation. HPH-mediated hydroxylation of Pro^{564} within the ODD of HIF-1α is required for the interaction of HIF-1α with pVHL. To test the hypothesis that EGCG inhibits HPH activity, *in vitro* HPH activity assays were performed. In this assay, HIF-1α ODD peptides that were bound to neutravidin beads via their biotin moieties were hydroxylated at Pro^{564} by recombinant HPH-2C. The prolyl-hydroxylated HIF-1α ODD peptide would then bind to ^{35}S pVHL. Thus, the radioactivity measured in this assay directly related to the amount of ^{35}S pVHL bound to the HIF-1α ODD peptide and the hydroxylation status of HIF-1α ODD peptide. As shown in Fig. 8A, the binding of ^{35}S pVHL to HIF-1α ODD peptide was dependent on the presence of recombinant HPH-2C and thus the hydroxylation of HIF-1α ODD peptide. However, EGCG treatment decreased the ^{35}S pVHL capture in a dose-dependent manner, with 40 μg/ml EGCG lowering the ^{35}S pVHL capture by almost 70%. On the other hand, EGCG had no significant effect on the interaction between ^{35}S pVHL and the positive control peptide containing HIF-1α ODD hydroxylated at Pro^{564} (Fig. 8B), indicating that EGCG did not exert any direct inhibitory effect on pVHL and HIF-1α ODD interaction. Thus, these data strongly support the hypothesis that EGCG has the ability to directly inhibit HIF-1α hydroxylation, thus preventing pVHL-HIF-1α ODD interaction.

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CHAPTER IV

DISCUSSION

This study provides the first evidence of EGCG, a major green tea polyphenol, inhibiting HIF-1α hydroxylation and subsequent HIF-1α degradation in normoxia, thus upregulating HIF-1-responsive genes including VEGF. Herein, we demonstrated a dose-dependent increase in VEGF promoter activity and VEGF protein secretion under normoxia, following EGCG treatment in prostate cancer cells (Figs. 1A and 1B). EGCG also increased HRE-mediated transcription. Although EGCG has been shown to inhibit proteasome activity (31), we did not observe accumulation of ubiquitinated HIF-1α after EGCG treatment. On the other hand, addition of exogenous ferrous ions abolished EGCG-mediated increase in VEGF expression and HRE-mediated transcription. The evidence that most tea polyphenols including EGCG are known to be metal ion chelators (25) and that HPH requires Fe^{2+} for its catalytic activity led to the hypothesis that EGCG might inhibit HPH activity. The data from figure 8 strongly supported the role of EGCG as a HPH activity inhibitor. Inhibition of pVHL and HIF-1α ODD peptide interaction indicated inhibition of prolyl hydroxylation by HPH in the presence of EGCG, since prolyl hydroxylation was a prerequisite to HIF-1α ODD and pVHL interaction. EGCG inhibited HIF-1α prolyl hydroxylation and the subsequent pVHL interaction, leading to HIF-1α protein stabilization and subsequent increase in HIF-1α transcriptional activity. A recent study by Zhou *et al.* (37) showed induction of HIF-1 activity by EGCG and epicatechin gallate (ECG) in T47D

breast cancer cells, in which ECG had a greater HIF-1 inducing effect. We have observed HIF-1 inducing activity by ECG in prostate cancer cells, but to a lesser degree than EGCG (data not shown).

Based on the results obtained, we propose a working model of EGCG's mechanism of action in increasing HIF-1 transcriptional activity. As shown in diagram 4, EGCG prevents HIF-1α hydroxylation by inhibiting HPH activity, this in turn leads to inhibition of HIF-1α and pVHL interaction, blocking HIF-1α ubiquitination and proteasomal degradation. Then, HIF-1α subunits dimerize with HIF-1β subunits and induce HIF-1-responsive genes such as VEGF. EGCG had no direct effect on HIF-1α-pVHL interaction as it did not inhibit binding of pVHL to HIF-1α ODD peptide containing hydroxylated Pro^{564}. Although EGCG has been shown to have proteasome inhibitory activity, our results demonstrated that EGCG acted prior to proteasome inhibition. Our results showed that there was no increase, but a modest decrease in ubiquitinated HIF-1α after EGCG treatment (Fig. 6). However, the possibility that EGCG still exerts proteasome inhibitory activity which may in part play a role in HIF-1α stabilization cannot be excluded.

Previously, EGCG has been shown to inhibit VEGF expression in breast (MDA-MB 231) (29) and colon cancer cells (30). The precise reasons as to why EGCG exhibits opposing effects on VEGF expression in different cancer cell lines are not clear. One possible explanation could be the difference in the basal level of expression of HIF-1α in these cancer cells. PC-3 and PC-3ML cells have a high amplification of the HIF-1α gene (40) and homozygous deletions of the PTEN gene (41) leading to upregulation of HIF-1α in a normoxic environment (42). These genetic changes could explain the relatively high basal levels of HIF-1α protein under normoxia. However, in breast cancer cells (MDA-MB231), HRE-mediated luciferase reporter gene activity and HIF-1α protein levels were undetectable in normoxia. Thus, in the absence of detectable

HIF-1α protein expression, EGCG had little effect in preventing HIF-1α protein degradation, if any.

The VEGF promoter possesses binding sites for the transcription factor, activator protein-1 (AP-1), whose binding has been demonstrated to potentiate HIF-1-mediated transcriptional activation of VEGF under hypoxia (43). EGCG decreased c-fos expression in breast cancer cells (MDA-MB231) (29), however, EGCG had no significant effect on c-fos expression in PC-3ML cells. This could explain the full transcriptional induction of the VEGF gene in PC-3ML cells following EGCG treatment. More studies are needed to further clarify EGCG's action in different cell types.

At present, the consequences of HIF-1α increase, following 24-h EGCG treatment, on the overall anticarcinogenic activity of EGCG is not clear. EGCG still induced apoptosis in PC-3ML cells after 48-h treatment. Thus, it is possible that HIF-1α increase may not play a significant role in the anticarcinogenic activity of EGCG. Nonetheless, it is more plausible to think that HIF-1α increase may attenuate apoptosis induced by EGCG, since HIF-1α regulates cell proliferaction and cell survival among its many other functions (1). Silencing of HIF-1α may prove to be beneficial in sensitizing prostate cancer cells to EGCG treatment.

In summary, this study demonstrated that EGCG upregulated HIF-1α transcription factor via inhibition of prolyl hydroxylation and subsequent pVHL interaction, leading to increase in VEGF promoter activity and protein expression. More studies are needed to further elucidate the significance of HIF-1α upregulation on the anticarcinogenic activity of EGCG.

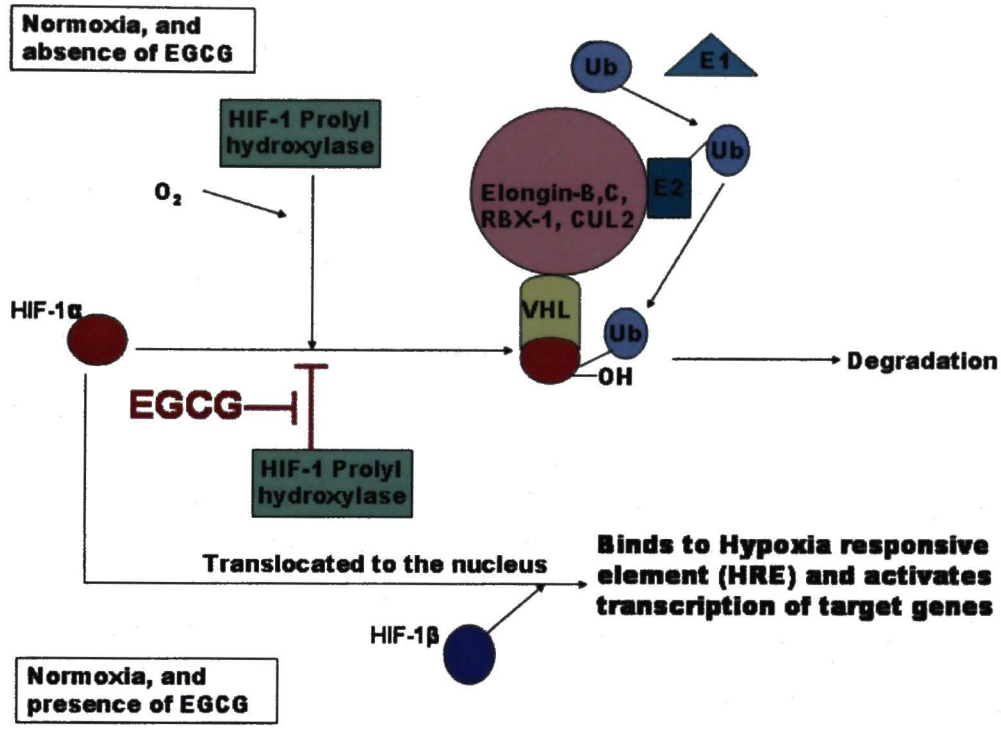

Diagram 4: Working model of EGCG's mechanism of action in increasing HIF-1 transcriptional activity. Under normoxic conditions and in the absence of EGCG, HIF-1α is hydroxylated at Pro^{402} and Pro^{564} within the HIF-1α ODD by Fe^{2+}-dependent HIF-1 prolyl hydroxylase, thereby allowing for HIF-1α recognition by an E3 ubiquitin ligase complex. pVHL, the substrate recognizing component of the E3 ubiquitin ligase complex, links the hydroxylated HIF-1α to the ubiquitination machinery and proteasome-mediated degradation. Under normoxic conditions and in the presence of EGCG, HIF-1 prolyl hydroxylase is inhibited by EGCG, thereby resulting in the accumulation of HIF-1α which translocates to the nucleus and associates with HIF-1β subunit. The HIF-1αβ heterodimer binds to hypoxia response element found in promoter/enhancer regions of HIF-1 target genes and activates their expression.

CHAPTER V

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CHAPTER VI

FIGURE LEGENDS AND FIGURES

Figure 1: EGCG increases VEGF promoter activity and VEGF protein expression under normoxia. (A) PC-3ML cells were transiently co-transfected with pGL3-hVEGF-Luc and pRL-TK plasmids and then treated with EGCG (0-40 μg/ml) in serum-free F-12K for 24 h. Luciferase activity was measured and normalized to the Renilla luciferase activity from the pRL-TK internal control. Data are the mean ± SD (n=3). * indicates $P<0.05$ versus EGCG 0 μg/ml. (B) PC-3ML cells were treated with EGCG (0-40 μg/ml) in serum-free F-12K for 24 h. Culture supernatants were harvested for VEGF ELISA. VEGF concentration in each well was normalized to the cell number in the same well. Data are the mean ± SD (n=3). * indicates $P<0.05$ versus EGCG 0 μg/ml.

Figure 2: EGCG upregulates HRE-mediated transcription under normoxia. PC-3ML cells were transiently co-transfected with pGL3-6xHRE-Luc and pRL-TK plasmids, and then treated with EGCG (0-40 μg/ml) in serum-free F-12K for 24 h. Luciferase activity was measured and normalized to the activity of the pRL-TK internal control. Data are the mean ± SD (n=3). * indicates $P<0.05$ versus EGCG 0 μg/ml.

Figure 3: EGCG increases HIF-1α protein levels. PC-3ML cells were treated with 20 μg/ml EGCG or 100 μM $CoCl_2$ in serum-free F-12K for 24 h. Cell lysates were prepared and analyzed for HIF-1α expression by Western blot analysis as described in Materials and Methods. Control and 20 μg/ml EGCG-treated PC-3ML cell lysates were also analyzed for HIF-1β expression. HIF-1α or HIF-1β expression in each sample was normalized to β–actin expression from the same sample and expressed as a fold change, with HIF-1α in control PC-3ML cells set at 1.

Figure 4: Dose-dependent HIF-1α accumulation after EGCG treatment. PC-3ML cells were treated with 0-40 μg/ml of EGCG in serum-free F-12K for 24 h. Whole cell lysates were prepared and analyzed for HIF-1α expression by immunoblot analysis. HIF-1α expression in each sample was normalized to β-actin density from the same sample and expressed as fold-change, with HIF-1α in control PC-3ML cells set at 1.

Figure 5: EGCG-mediated decrease in ubiquitinated HIF-1α. PC-3ML cells were transiently transfected with pFLAG-HIF-1α plasmid and then treated with or without EGCG (20 μg/ml) in serum-free F-12K medium for 24 h. Whole cell lysates were prepared and FLAG-HIF-1α fusion protein was immunoprecipitated from the cell lysates with anti-FLAG antibody. Accumulation of HIF-1α and ubiquitinated HIF-1α in the immunoprecipitates was determined by immunoblot analysis with antibodies to HIF-1α and ubiquitin respectively.

Figure 6: Fe^{2+} abolishes EGCG-induced increase in HRE-mediated transcription. PC-3ML cells were transiently co-transfected with pGL3-6xHRE-Luc and pRL-TK plasmids and treated with none, $FeSO_4$ alone, EGCG (0-40 μg/ml) ± 300 μM $FeSO_4$, or DFX (100 μM) ± 300 μM

$FeSO_4$ in serum-free F-12K for 24 h. Luciferase activity was measured and normalized to the activity of the pRL-TK internal control. Data are the mean ± SD (n=3). * indicates $P<0.05$ versus EGCG 0 μg/ml.

Figure 7: Fe^{2+} abolishes EGCG-induced increase in VEGF expression. PC-3ML cells were treated with or without EGCG (0-40 μg/ml) ± 300 μM $FeSO_4$ in serum-free F-12K for 24 h. Culture supernatants were harvested for VEGF ELISA. $VEGF_{165}$ concentration in each well was normalized to the cell number in each well. Data are the mean ± SD (n=3). * indicates $P<0.05$ versus EGCG 0 μg/ml.

Figure 8: EGCG inhibits in-vitro HPH activity. Synthetic biotinylated peptides corresponding to (A) HIF-1α C-terminal ODD (556-574) or (B) Pro^{564} hydroxylated HIF-1α C-terminal ODD (556-574) were bound to neutravidin beads and incubated with recombinant HPH-2C for 1 h in the absence or presence of EGCG (10-40 μg/ml), followed by a 20-min incubation with ^{35}S pVHL. ^{35}S pVHL bound to the peptide was measured by scintillation counting. Data are the mean ± SD (n=2). * indicates $P<0.05$ versus reaction containing 0.5 μg HPH and no EGCG.

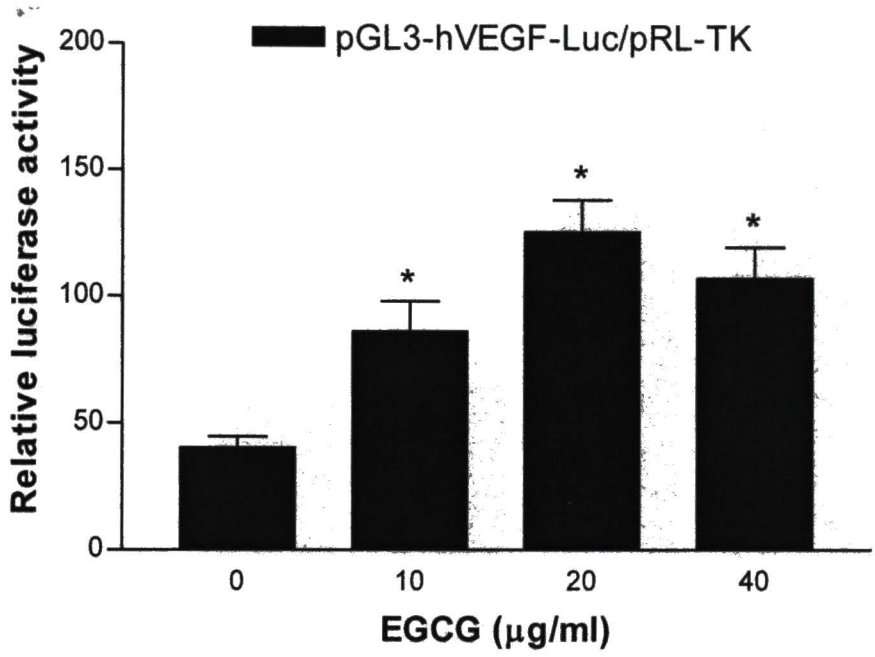

Figure 1A: EGCG-mediated increase in VEGF promoter activity under normoxia.

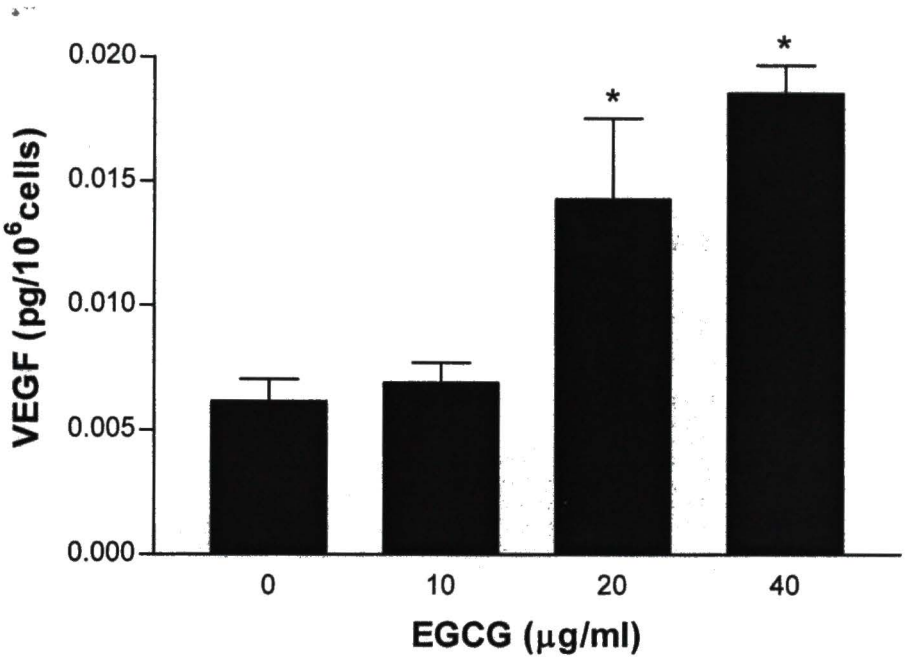

Figure 1B: EGCG increases VEGF protein expression under normoxia.

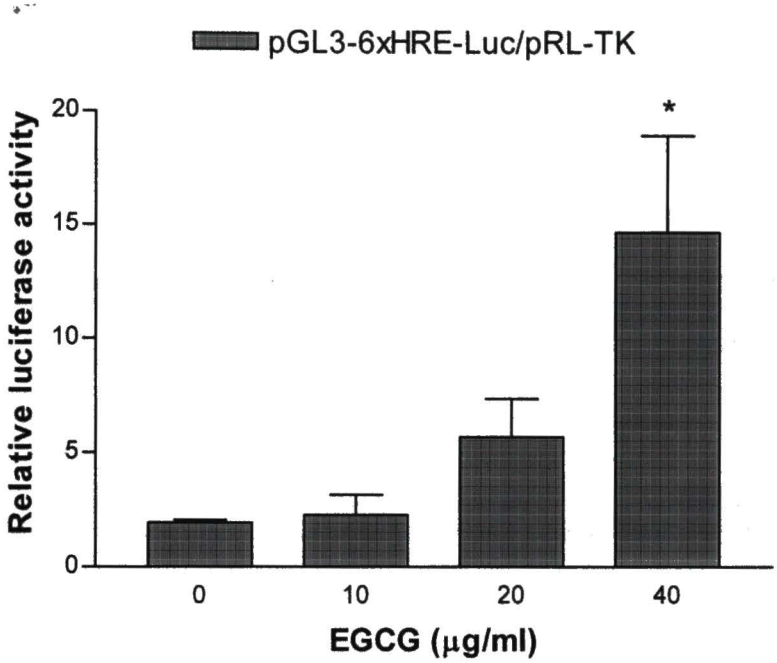

Figure 2: EGCG upregulates HRE-mediated transcription under normoxia.

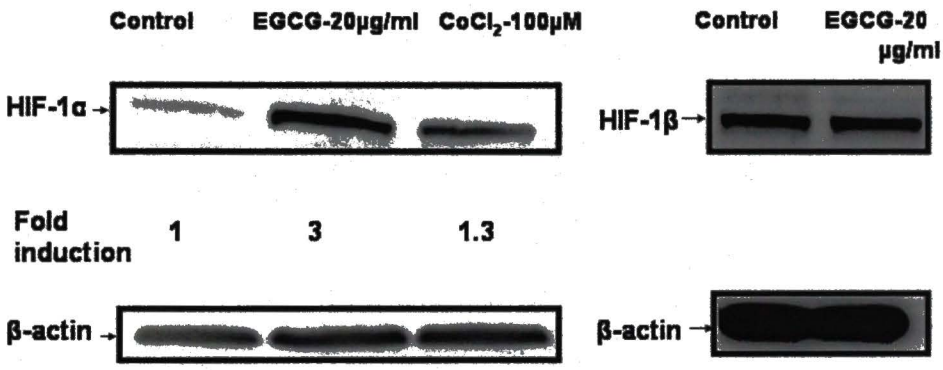

Figure 3: EGCG increases HIF-1α protein levels.

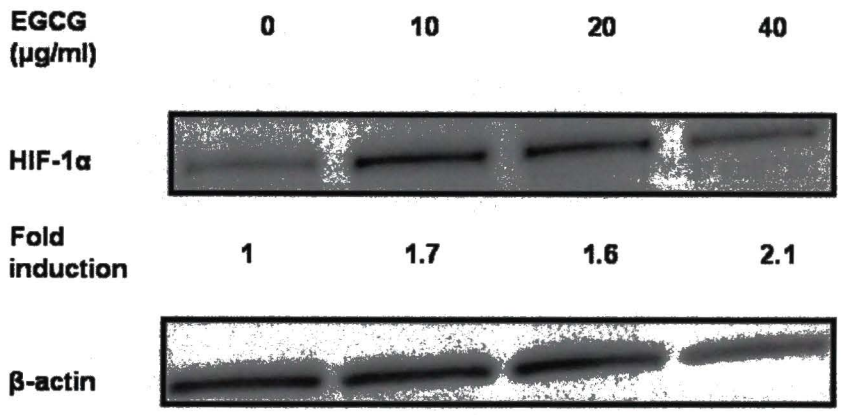

Figure 4: Dose-dependent HIF-1α accumulation after EGCG treatment.

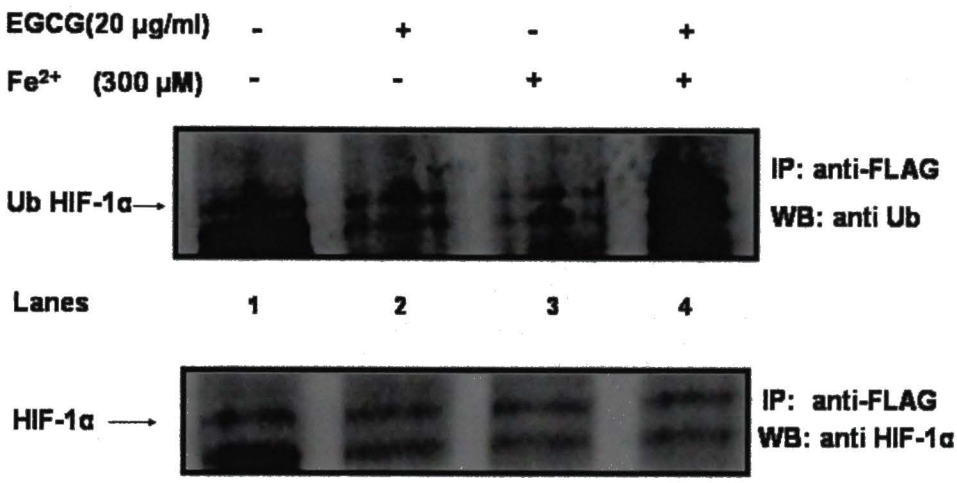

Figure 5: EGCG-mediated decrease in ubiquitinated HIF-1α, and accumulation of ubiquitinated HIF-1α after co-incubation with EGCG and $FeSO_4$.

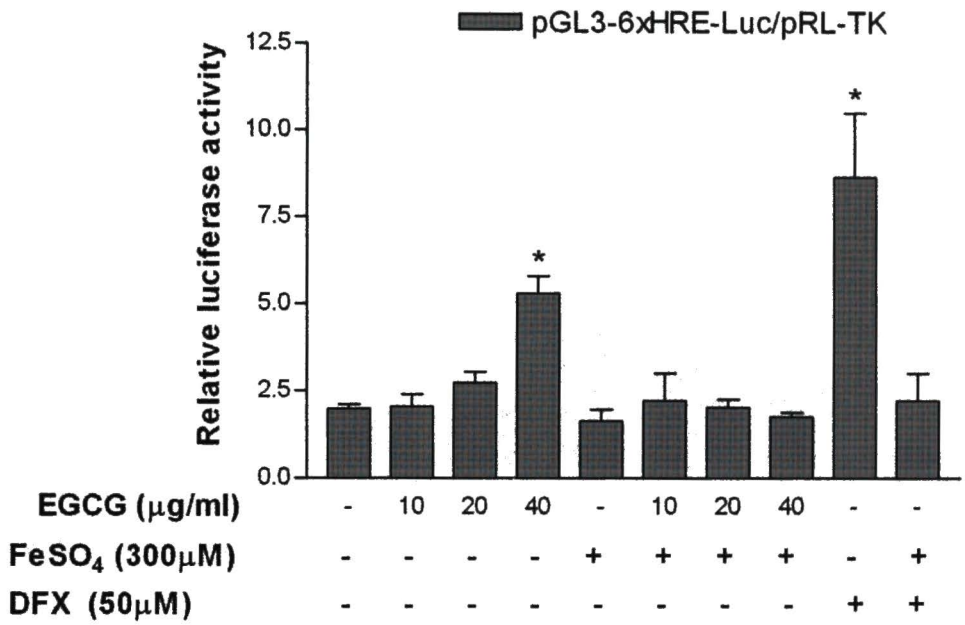

Figure 6: Fe^{2+} abolishes EGCG-induced increase in HRE-mediated transcription.

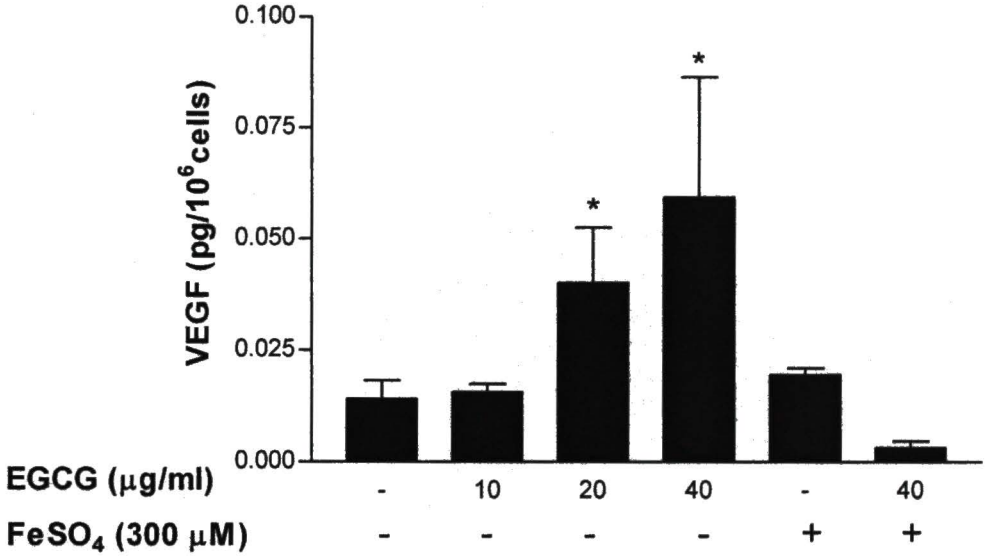

Figure 7: Fe^{2+} abolishes EGCG-induced increase in VEGF expression.

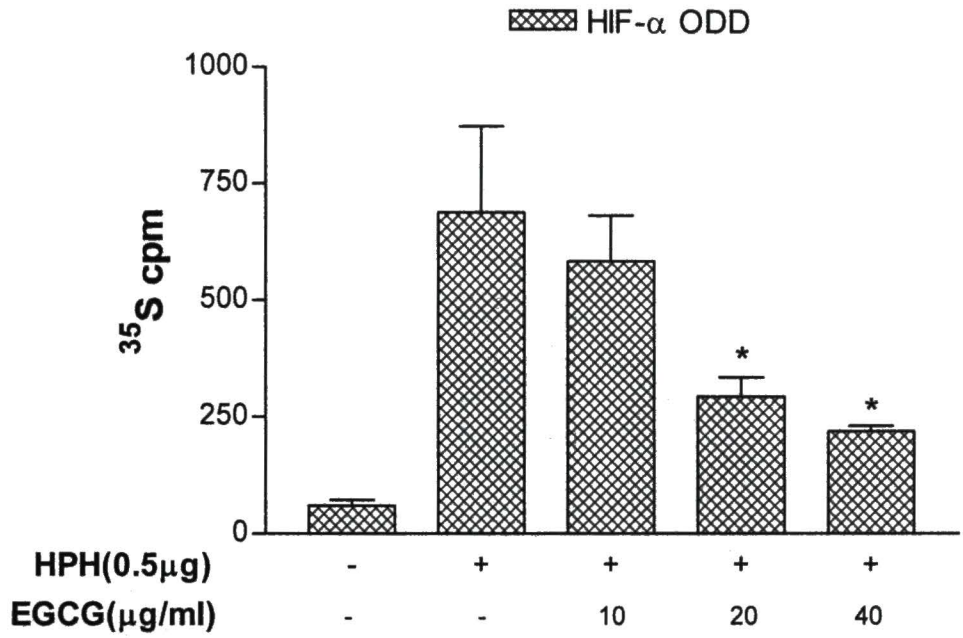

Figure 8A: EGCG inhibits in-vitro HPH activity.

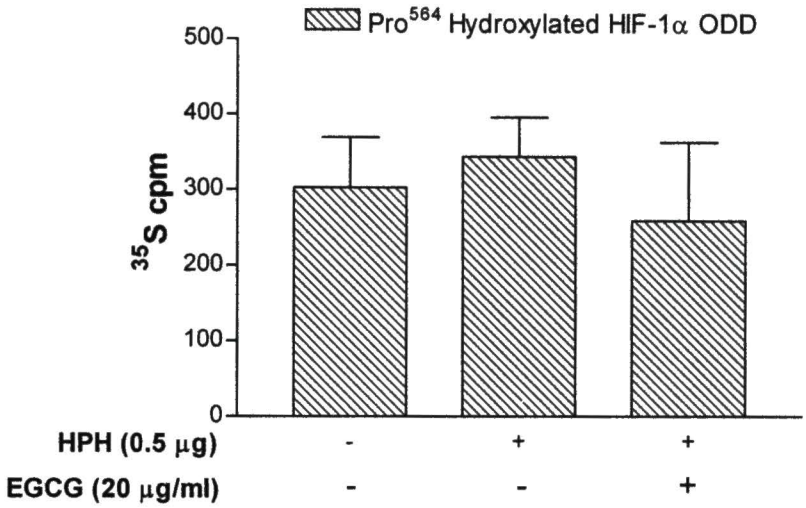

Figure 8B: EGCG does not directly inhibit pVHL-HIF-1α ODD interaction.