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Gensenoside Rg3 Inhibits Hypoxia-induced VEGF Expression in Human Cancer Cells

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Key Words

Ginsenoside Rg3 • VEGF • Angiogenesis • Hypoxia • Cancer

Abstract

Aims: The ginsenoside Rg3 (Rg3) inhibits xenograft growth and angiogenesis in tumors mainly via down-regulates VEGF expression. This study was designed to investigate the mechanisms by which Rg3 down-regulates VEGF expression. Methods: MTT assay was performed to investigate the effect of Rg3 on the growth of human esophageal carcinoma cell line Eca-109 and 786-0 cells under normoxic and hypoxic conditions. ELISA was used to detect VEGF protein secreted by the cells under normoxic and hypoxic conditions. Real-time quantitative reverse transcriptase polymerase chain reaction and Western blotting were used to detect gene expression and protein synthesis. Results: Rg3 inhibited Eca-109 and 786-0 cell proliferation and induced a significant reduction in VEGF mRNA under hypoxia conditions. Rg3 treatment inhibited hypoxia-induced expression HIF-1α, COX-2 and NF-κB under normoxic and hypoxic conditions. Treatment with Rg3 reduced the hypoxia-induced phosphorylation of STAT3 in a dose-dependent manner in the both cell lines. Rg3 treatment also inhibited the phosphorylation of ERK1/2 and JNK induced by hypoxia. Conclusions: Rg3 targets hypoxia-induced multiple signaling pathways to down-regulate VEGF expression in cancer cells. These actions may contribute to the overall efficacy of Rg3 against tumor angiogenesis and growth.

Introduction

Ginseng has been used as a medicinal plant for the treatment and prevention of various diseases, including cancer [1]. The ginsenosides are apparently responsible for most of the pharmacological effects of ginseng. The ginsenoside Rg3 (Rg3) is one of ginsenosides that assert the anticancer properties. Previous studies have revealed pleiotropic capabilities of Rg3 against various cancers. Rg3 treatment induces growth inhibition, cell cycle arrest and apoptotic death in prostate carcinoma LNCaP cell line [2]. Rg3 inhibits tumor growth and progression via

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inhibition of xenograft growth and angiogenesis [3]. It also inhibits metastasis and enhances the susceptibility of colon cancer cells to docetaxel and other chemotherapeutics [4-5]. Therefore Rg3 could be useful for prevention or treatment for human cancer.

A growing body of evidence indicates that vascular endothelial growth factor (VEGF) contributes to tumor progression, angiogenesis and metastasis. Decreased VEGF expression may contribute to antitumor effects of some chemotherapeutic agents [6-8]. Although Rg3 inhibits human tumor xenografts growth and angiogenesis in xenografts tissues [3, 9], the effect of Rg3 on VEGF expression, in particular under hypoxic conditions, is unknown. One of the master regulators that orchestrate the cellular responses to hypoxia is hypoxia inducible factor-1 (HIF-1) [10]. HIF-1 is a heterodimer composed of an inducible HIF-1α subunit and a constitutively expressed HIF-1β subunit. HIF-1 biologic activity depends on the function of HIF-1α subunit, which is tightly regulated by oxygen tension [11]. HIF-1α protein subunits are inducibly overexpressed in human tumors and tumor cell lines and have closely relation with angiogenesis, tumor growth and metastasis [12, 13]. In preclinical and clinical studies, the inhibition of HIF-1α activity had reduced tumor growth [14]. Most experimental show that the overexpression of VEGF can be regulated at the transcriptional level by HIF-1α, thus make itself an appropriate anticancer target for inhibition of neoangiogenesis.

Another principal regulator of VEGF expression is Cyclooxygenase-2 (COX-2), an inducible enzyme that catalyzes the conversion of arachidonic acid to bioactive lipids including prostaglandins and thromboxanes, is also induced in response to hypoxia and other stressors [15]. Overexpression COX-2 and resultant prostaglandins promote cancer development and propagation through multiple mechanisms, including enhancement of angiogenesis, stimulation of growth, migration, invasiveness, and resistance to apoptosis [16]. High expression of VEGF is associated with the up-regulation of COX-2 expression in various tumors, suggesting that these two genes may have close relation [17].

The transcription factor Nuclear Factor kappa B (NF-κB) is a major regulator of oncogenes expression. The most abundant form of this protein is a heterodimer of p50 and p65 subunits in tumor cells, which form an inactive complex with IκBα, its inhibitor in the cytoplasm. Upon certain cellular stimulation, IκBα, protein is phosphorylated and subsequently degraded. The IκBα, degradation and the p65 subunit phosphorylation result in high transactivation potential of NF-κB [18]. The role of NF-κB on tumor promoting has been well-documented through the transcriptional upregulation of a number of cytokines, adhesion molecules, growth factors, oncogenes, antiapoptotic proteins, some proapoptotic factors, and even certain viral genes. A considerable number of studies suggested that NF-κB and its activating signaling molecules may act as tumor suppressors under some circumstances [19, 20]. Recently, the role of NF-κB in tumor angiogenesis has attracted more attention [21]. Given the central role of NF-κB and hypoxia in tumor growth and angiogenesis, and because the functional relation between NF-κB and VEGF during hypoxia has been established, our aim was to investigate the role of NF-κB in inhibiting hypoxia-induced VEGF by Rg3.

A number of studies have reported cross-talk between VEGF and the signal pathways of the transcription factor signal transducer and activator of transcription 3 (STAT3) and mitogen activated protein kinase (MAPK), and the members of the STAT3 and MAPKs are involved in the process of angiogenesis [22-25]. Whatever the STAT3 or the three major MAPK: extracellular signal-regulated kinase (ERK1/2), the c-jun N-terminal kinase or stress-activated protein kinases (JNK), and the p38 MAP kinases (p38), all can be induced by hypoxia. These factors, which may play an important role in tumor angiogenesis, have drawn attention as another target signal transduction pathway to inhibit tumor growth and metastasis via decrease of VEGF expression [24-26].

In this study, we sought to investigate the effect of Rg3 on VEGF expression in human esophageal carcinoma cell line Eca-109, and in renal cell carcinoma cell line 786-0 cells. The signalling pathways involved in the Rg3's effect were also explored.

Materials and Methods

Reagents

The following primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): rabbit polyclonal antibody to human β-actin (sc-7210), rabbit polyclonal antibody to human HIF-1α (sc-10790), rabbit polyclonal antibody to human COX-2(sc-7951), rabbit polyclonal antibody to human p65 (SC-7151), rabbit polyclonal antibody to human STAT3 (sc-7179), rabbit polyclonal antibody to human phosphorylated $STAT3^{Tyr705}$ (sc-135649), rabbit polyclonal antibody to human phosphorylated p38 (sc-7149), rabbit polyclonal antibody to human p38 (sc-17852-R), mouse monoclonal antibody ERK1/2 (sc-135900), rabbit polyclonal antibody to human phosphorylated ERK1/2 (sc-16982), mouse monoclonal

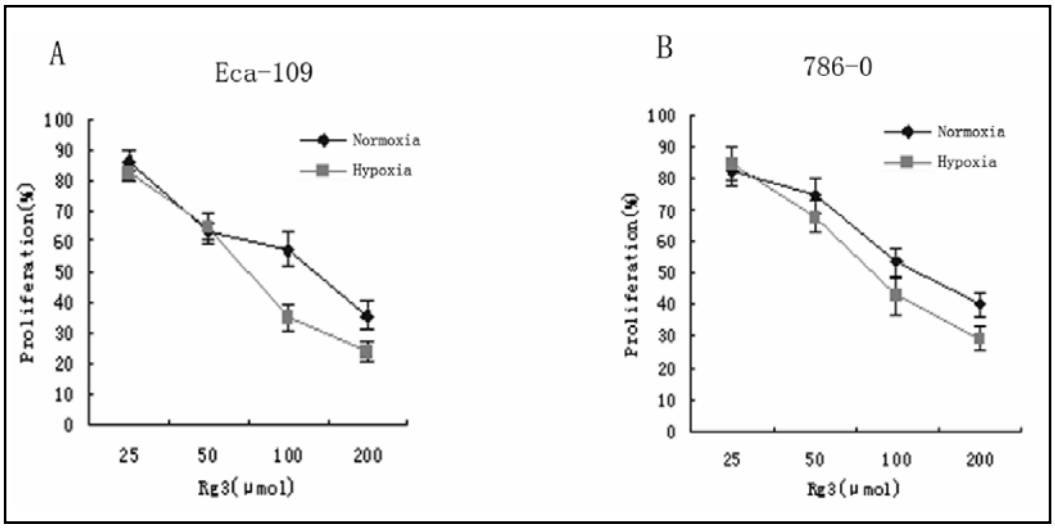


Fig. 1. Effects of Rg3 on the proliferation of Eca-109 and 786-0 cells.

antibody to human phosphorylated JNK (sc-6254), and rabbit polyclonal antibody to human JNK (sc-572).

Rg3 (Yatai Pharmaceutical Co., Ltd, Jilin, China) was dissolved in distilled water at a concentration of 100 mmol/L. AG490, SB203580, PD98059, SP600125 were purchased from Calbiochem (San Diego, CA). Proteasome inhibitors, MG132, and HIF-1α protein synthesis inhibitor, cycloheximide, were obtained from Sigma (St. Louis, MO) and all were dissolved in DMSO, stored at -80°C as a stock solution. The final DMSO solution concentration did not exceed 0.5% throughout the study. The following primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): rabbit polyclonal antibody to human β-actin, rabbit polyclonal antibody to human HIF-1α, rabbit polyclonal antibody to human COX-2, rabbit polyclonal antibody to human p65, rabbit polyclonal antibody to human STAT3, rabbit polyclonal antibody to human phosphorylated $STAT3^{Tyr705}$, rabbit polyclonal antibody to human phosphorylated p38, mouse monoclonal antibody ERK1/2, rabbit polyclonal antibody to human phosphorylated ERK1/2, mouse monoclonal antibody to human phosphorylated JNK, and rabbit polyclonal antibody to human JNK.

Cell lines

The human esophageal carcinoma cell line Eca-109 and renal cell carcinoma cell line 786-0 cells were cultured in RPMI 1640 (GIBCO). The cells were cultured at 37°C in a humidified atmosphere, and 5% CO_2 supplemented with 10% fetal bovine serum (GIBCO) and antibiotics. For hypoxic treatment, the cells were cultured in a temperature- and humidity-controlled environmental chamber containing 1%O_2, 5% CO_2, and 95% N_2.

MTT assay

MTT assay was performed to investigate the effect of Rg3 on the growth of Eca-109 and 786-0 cells under normoxic and hypoxic conditions. Cells were seeded in a 96-well plate at a density of $7.5x10^3$ cells/well continuing cultured for 24 h. The cells were incubated in hypoxic environment with medium plus Rg3 at 25, 50, 100, 200 μmol/ml for another 48 h. Thereafter, 20 μL of MTT (5 g/L, Sigma) was added to each well and further incubated for 4 h. Finally, the culture medium was removed and 150 μL of DMSO was added to each well. The absorbance was determined with a spectrophotometer reader at 570 nm. The inhibition rates of cell growth were calculated according to the formula: inhibition rate (%) = (1−mean absorbance of treated group/mean absorbance of untreated group) x 100%. The IC_{50} values were derived from the dose-response curves.

VEGF ELISA assay

To detect VEGF protein secreted by the cells in the medium under normoxic and hypoxic condition after a short period of time (48h) cells were treated with Rg3 at different concentration corresponding to the MTT experiment. At the end of the experiment, the cells were calculated and medium harvested and immediately frozen with liquid nitrogen. Each sample was analyzed by a VEGF ELISA kit (Nanjing Keygen Biotech, China) according to the manufacturer's guidelines.

Real-time quantitative reverse transcriptase polymerase chain reaction

Total RNA was isolated from the cells using the trizol (Sigma, USA), according to the manufacturer's protocol and verified by 1% gel electropherogram and A260/A280. cDNA was prepared using the Rever Aid TM First Strand cDNA Synthesis Kit (Takara Biotech, Dalian, China) following the instructional protocols. PCR primers were designed and tested for gene homology using the Primer Premier 5.0 and BLAST (www.ncbi.nlm.nih.gov/BLAST) programs. Primer sequences were as follows: VEGF forward 5'-TTC GGA GGA GCC GTG GTC CG-3' and reverse 5'-GTC CAT GAG CCC GGC TTC CG-3', Hif-1α forward 5'-GGC AGC AACGAC ACA GAA ACT GA-3' and reverse 5'-TTG GCG TTT CAG CGG TGG GT-3', β-actin forward 5'-GCC CTG GCA CCC AGC ACA AT-3' and reverse 5'-GAC GAT GGA GGG GCC GGA CT-3'. Gene expression was quantified with the Roche Light Cycler SYBR Green I kit (Tiangen Biotech, Beijing, China) and samples were run on the ABI7300 HT Fast Real Time PCR System from Applied Biosciences (USA). Within each experiment, samples were run in triplicate.

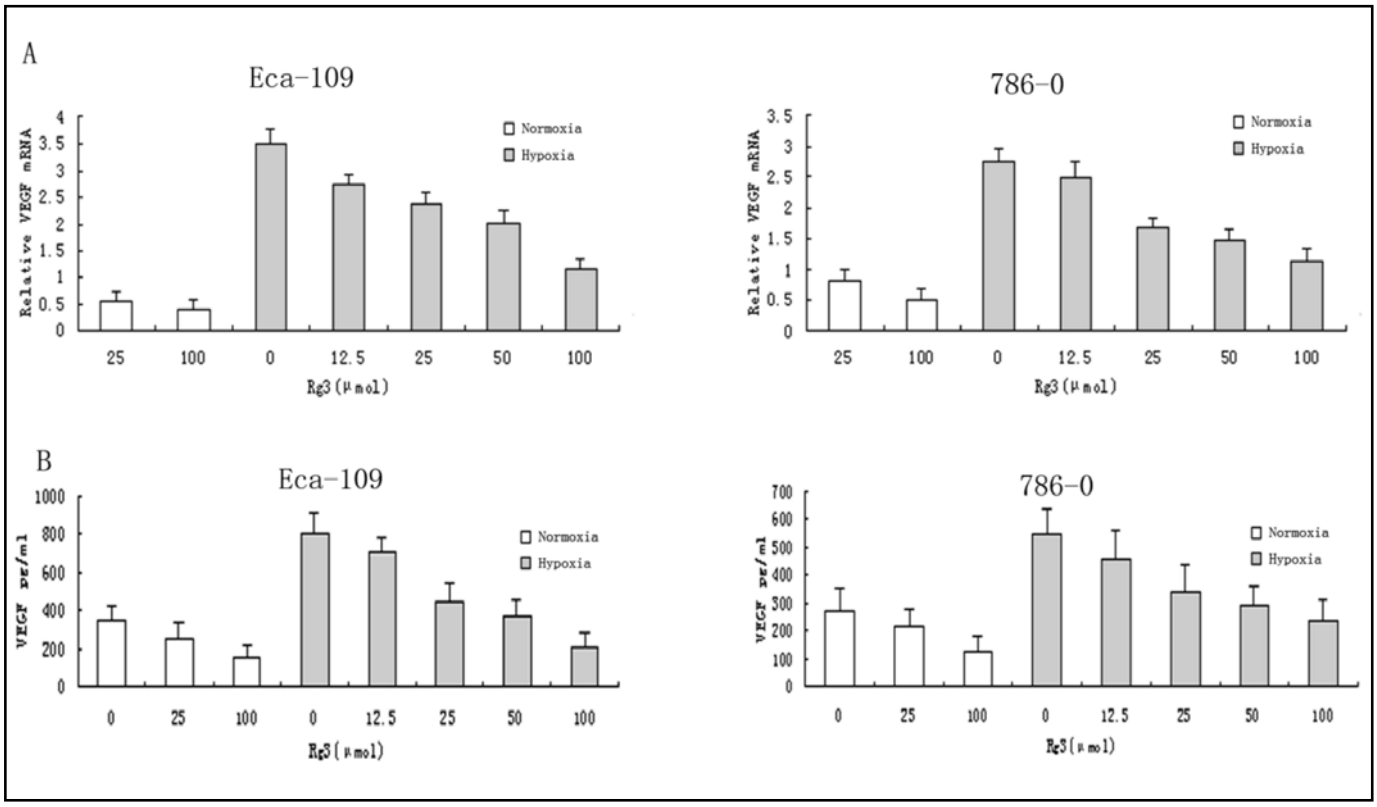


Fig. 2. Inhibition of the VEGF expression by Rg3. A. Total RNA was extracted and analyzed by Real-time PCR after 24 h treatment. B. VEGF protein concentrations in the medium were measured by ELISA assay after 24 h treatment.

Western blot analysis

Cells were cultured in 6-well plates to near confluence, treated with Rg3 and placed in hypoxic (1% O_2) incubators for indicated time points. Cells were immediately harvested in lysis buffer on the ice. Total protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, CA, USA). Proteins (30 μg/lane) were separated on 10% SDS-PAGE (BioRad) and transferred to PVDF membranes, using an electrophoretic transfer instrument (Bio-Rad). Membranes were immunoblotted with 1:2000 dilution of HIF-1α, COX-2, p65, pSTAT3, STAT3, pp38, p38, pERK1/2, ERK1/2, pJNK, JNK and 1:5000 of β-actin antibodies overnight. After washing in TBST, membranes were incubated with a 1:2000 dilution of peroxidase-conjugated anti-rabbit IgG, and visualized using the Super Signal West Dura Extended Duration Substrate (Pierce). All experiments were done in triplicate. Films of proteins were developed and scanned, and bands were analyzed as a ratio of target protein/ β-actin control using the Scion Image for Windows program, version 4.02.

Statistical analysis

Data are presented as mean ± SD. The significance of differences groups was assessed using One-way ANOVA. *P* values less than 0.05 were considered significant.

Results

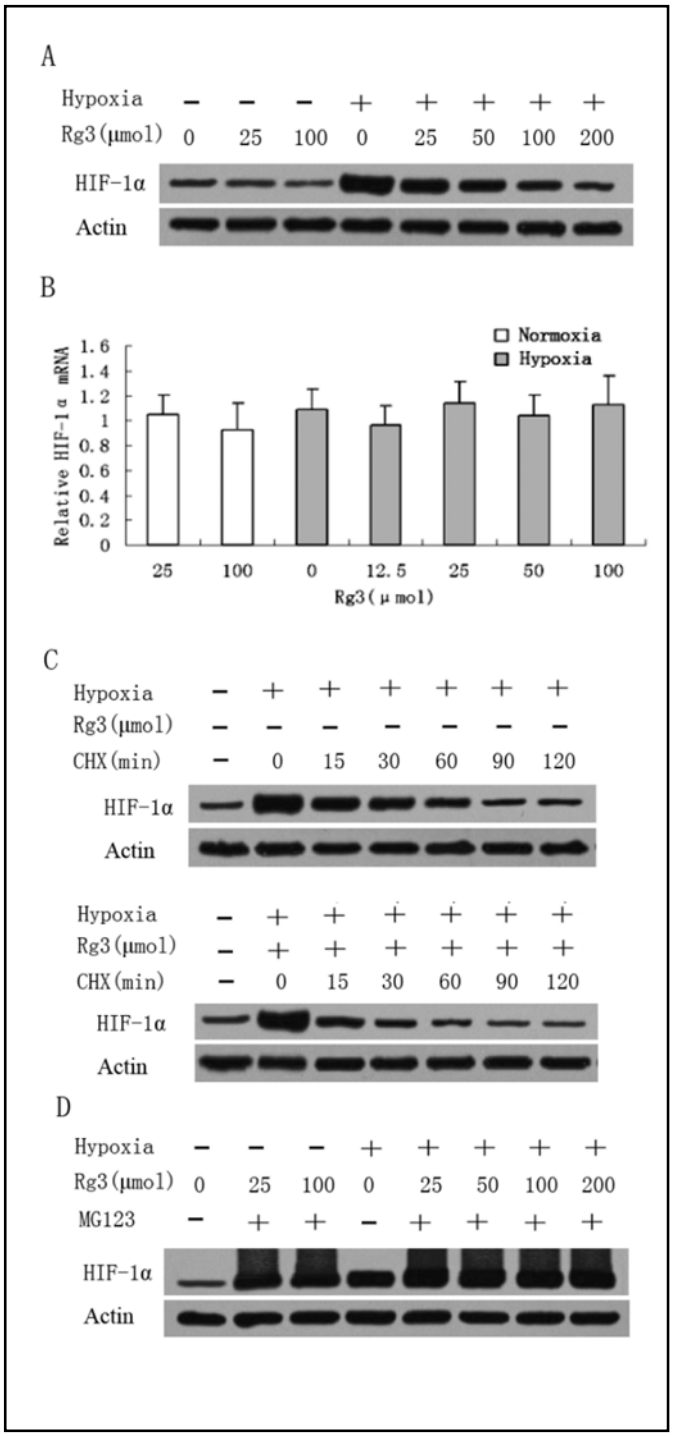
Effect of Rg3 on Eca-109 and 786-0 cell proliferation

As shown in Fig. 1, the cell proliferation of Eca-109 and 786-0 cells at 50, 100 and 200 μmol of Rg3 was significantly lower than at 25 μmol of Rg3 (P <0.01). The cell proliferation under hypoxic condition was significantly lower than under normoxic conditions at 100 or 200 μmol of Rg3 (P <0.01).

Effects of Rg3 on VEGF activation

As shown in Fig. 2, under normoxia conditions, there was a small but significant reduction in VEGF mRNA in both Eca-109 and 786-0 cell lines, after treatment with Rg3 at 100 μmol (P <0.05). Significant reduction in VEGF mRNA was observed in all Rg3 concentrations under hypoxia conditions (P <0.01). VEGF concentrationswere reduced at all Rg3 concentrations under both hypoxia and normoxia conditions in both cell lines (P <0.01).

Fig. 3. Effect of Rg3 on hypoxia-induced HIF-1α protein stability in Eca-109 cells. A and B: Eca-109 cells were incubated in various concentrations of Rg3 for 16 h. C: Eca-109 cells were treated with solvent alone or 100 μmol/L Rg3 for 16 h under hypoxic conditions. D: Eca-109 cells were pretreated with solvent alone or 20 μmmol MG132 (*MG*) for 30 min, followed by treatment with Rg3 for 6 h.



Effects of Rg3 on HIF-1α activation

Eca-109 cells were incubated in various concentrations of Rg3 for 16 h under normoxic and hypoxic conditions. As shown in Fig. 3, Rg3 reduced HIF-1α protein but not HIF-1α mRNA in Eca-109 cells. To determine if Rg3 can promote HIF-1α degradation, Eca-109 cells were cultured in complete medium and then pretreated with solvent alone or Rg3 for 16 h, followed by incubation with cycloheximide to block ongoing protein synthesis. HIF-1α protein levels were examined by western blot at various time intervals. In the presence of cycloheximide, the degradation of HIF-1α protein was faster in cells treated with Rg3 than in cells treated with solvent alone (Fig. 3C). Because HIF-1α protein is degraded mainly through the ubiquitin-proteasome pathway, and ubiquitinated HIF-1α is immediately degraded by proteasome, cells were treated with proteasome inhibitor MG132 in the presence or absence of Rg3 for 6 h. Treatment of the cells with MG132 increased HIF-1α protein ubiquitination and total HIF-1α protein levels, and Rg3-induced HIF-1α decrease was prevented completely by MG132 (Fig. 3D).

Effects of Rg3 on COX-2 Activation

To determine whether the effect of Rg3 on VEGF expression might be due to COX-2, Eca-109 and 786-0 cells were treated with various concentrations of celecoxib, a COX-2 inhibitor, for 24 h. As shown in Fig. 4, celecoxib reduced VEGF expression under normoxic and hypoxic conditions (P <0.01, Fig. 4A). Eca-109 and 786-0 cells were also treated with various concentrations of Rg3 for 24 h. Hypoxia induced high levels of COX-2 protein expression in Eca-109 and 786-0 cells, however, this effects was abolished by pretreatment with Rg3 (Fig. 4B).

Effects of Rg3 on NF-κB Activation

NF-κB transcription factor is activated by hypoxia and which mediates many processes involved in tumor angiogenesis [27]. In this regard, because VEGF could also be transcriptionally activated by NF-κB, we first detect the effect of NF-κB inhibitor PDTC on VEGF expression in Eca-109 and 786-0 cells. As shown in Fig. 5A, PDTC down-regulated VEGF expression under normoxic and hypoxic conditions (P <0.01). We next

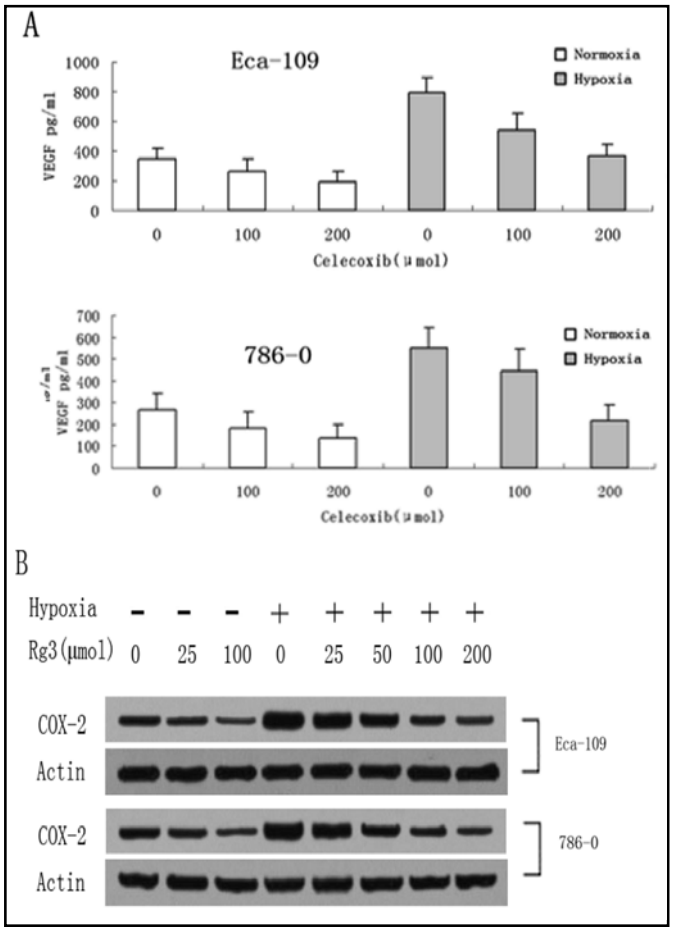


Fig. 4. Rg3 downregulated VEGF expression via COX-2 pathway. A. Eca-109 and 786-0 cells were pretreated with various doses of COX-2 inhibitor celecoxib for 30 min, followed by incubation under normoxic and hypoxic conditions for 24 h. B. Eca-109 and 786-0 cells were treated with various doses of Rg3 and incubated under normoxic and hypoxic conditions for 24 h. COX-2 protein were analyzed by Western blot.

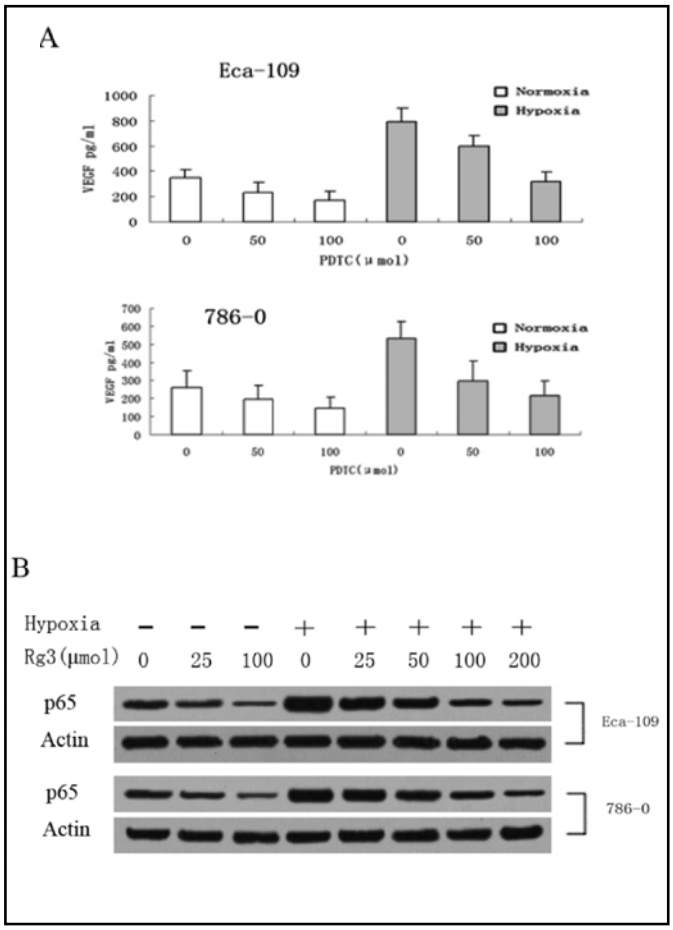


Fig. 5. The Effect of Rg3 on NF-κB signaling.A. Eca-109 and 786-0 cells were pretreated with various doses of NF-κB inhibitor PDTC under normoxic and hypoxic conditions for 24 h. Cell medium were subjected to ELISA for VEGF analysis. B. Eca-109 and 786-0 cells were incubated in various concentrations of Rg3 under normoxic and hypoxic conditions for 24 h. Western blot was used to examine p65 expression.

assessed Rg3's effect on NF-κB activities by determining Rg3 effect on p65, the activated subunit form of NF-κB. Eca-109 and 786-0 cells were treated with various concentrations of Rg3 under hypoxic conditions for 24 h. Rg3 inhibited hypoxia induced p65 expression (Fig. 5B).

Effects of Rg3 on STAT3 and MAPKs activation

Eca-109 and 786-0 cells were treated with STAT3 and MAPKs signaling inhibitors to investigate the role of STAT3 and MAPKs signaling pathway in VEGF expression. These inhibitors reduced VEGF expression under normoxic and hypoxic conditions (P <0.01, Fig. 6A). Treatment with Rg3 reduced the hypoxia-induced phosphorylation of STAT3 in a dose-dependent manner in the both cell lines (P <0.01, Fig. 6B). Rg3 treatment also inhibited the phosphorylation of ERK1/2 and JNK induced by hypoxia, but have no effect on phosphorylation of p38 (Fig. 6. B).

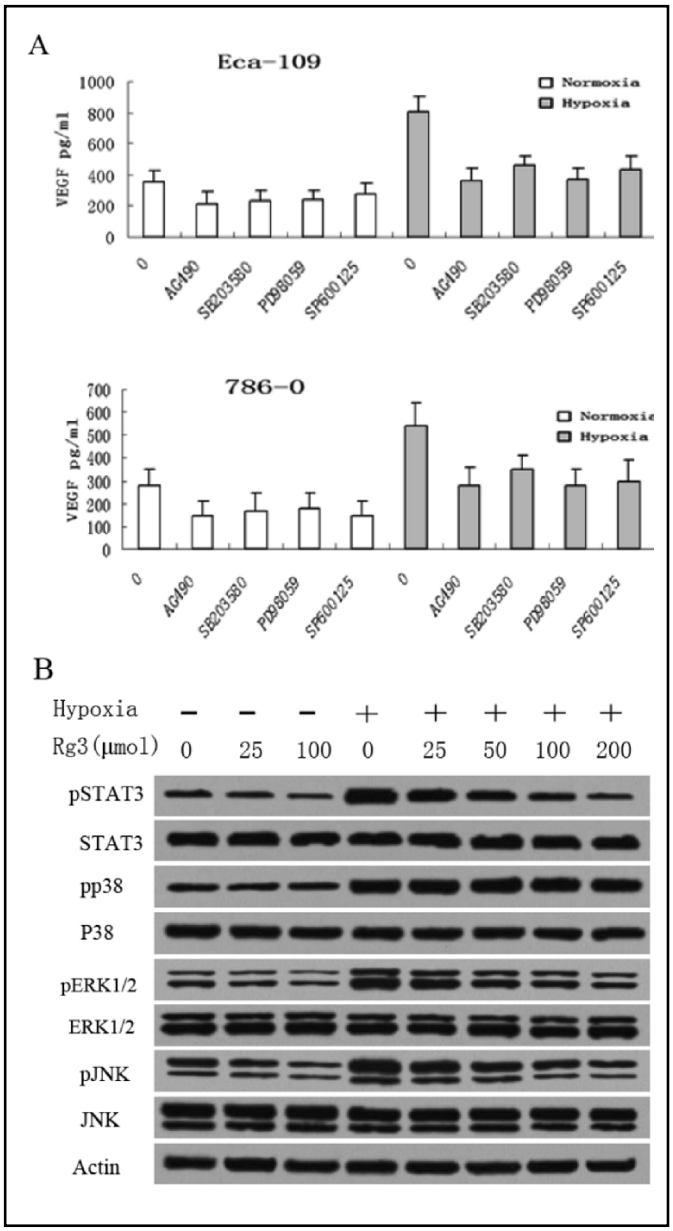


Fig. 6. The Effect of Rg3 on hypoxia-induced phosphorylation of STAT3 and MAPKS in Eca-109 and 786-0 cells. A. Eca-109 and 786-0 cells were treated with AG490 (100 μmol), SB203580 (50 μmol), PD98059 (50 μmol), SP600125 (40 μmol) under normoxic and hypoxic for 24 h. Cell medium were subjected to ELISA for VEGF analysis. B. Eca-109 and 786-0 cells were incubated in various concentrations of Rg3 under normoxic and hypoxic for 6h, Western blot was used to examine the changes in phosphorylation of STAT3, p38, ERK1/2 and JNK.

Discussion

Hypoxia commonly develops within solid tumors because tumor cell proliferation is faster than the rate of new blood vessels formation, and thus leads to a series of pathological and physiological changes [28]. Clinical and experimental evidence suggests that the most significant of those changes is that hypoxia stimulates VEGF expression in tumors and promotes angiogenesis to meet the metabolic requirement for rapidly tumor growth [29]. Thus, VEGF, a key angiogenic factor, plays an important role in tumor angiogenesis and progression. Previous experimental and clinical data show that VEGF mRNA and protein are highly expressed in various human tumors and tumor cell lines. VEGF has been well established to have a crucial role in angiogenesis and tumor progression. The inhibition of VEGF expression and its receptor function dramatically blocked tumor growth, invasion, and metastasis *in vitro* and *vivo* studies [6-8]. Thus, the potential capacity of inhibiting angiogenesis by targeting VEGF is an important development in identifying a novel chemotherapeutic approach in treating human tumors. Moreover, those factors that directly or indirectly regulate hypoxia-stimulated angiogenesis represent potential anticancer therapeutic targets.

Recently, it was reported that Rg3 targeting VEGF blocks tumor growth [3, 9]. We investigated whether Rg3 have the same effect on Eca-109 and 786-0 cells. We demonstrated in this study that Rg3 inhibited VEGF expression at transcriptional level and post-transcriptional level in two cells lines under both hypoxic and normoxic conditions (Fig. 1). These data suggest that not only did Rg3 suppress VEGF expression under normoxia, it also suppressed the enhanced VEGF expression under hypoxia. They also suggest that Rg3 maybe just functions as a VEGF inhibitor in most cases but not specifically under hypoxia.

Increased VEGF expression can be induced by hypoxia and this induction is believed to be mediated primarily through HIF-1α. HIF-1α binds to a hypoxia-responsive element (HRE) of the VEGF promoter and activates expression of the VEGF gene at the transcriptional level [30]. HIF-1α also activates the transcription of other genes involved in crucial aspects of cancer angiogenesis, such as basic fibroblast growth factor (bFGF) [31]. HIF-1α is over-expressed in many human cancers and the levels of HIF-1α in cells correlate with microvessel density [32]. In the current study, we have demonstrated that Rg3 inhibits the up-regulation of HIF-1 α expression induced by hypoxia in human colon cancer cells. In particular, we have shown that the HIF-1α protein but not the HIF-1α mRNA is decreased by Rg3.

It is known that the HIF-1α protein is rapidly degraded by the ubiquitin/proteasome pathway under aerobic conditions [33, 34]. Under hypoxic conditions, the ubiquitination of HIF-1α protein is inhibited and this results in the up-regulation of HIF-1α protein stability. We found that Rg3 promoted HIF-1α protein degradation and significantly shorten the half-life of HIF-1 α protein (Fig. 3). Furthermore, this degradation procession was through the 26 proteasome degradation pathway. These data suggest that Rg3 affects HIF-1α protein stability partly through promoting its degradation. HIF-1α was greatly induced by hypoxia and dramatically decreased when treated with Rg3 in human cancer cell line, combined with other results that the HIF-1α is a key regulator of VEGF expression, showing that Rg3 decreased VEGF expression via blocking HIF-1α pathway. Although 786-0 cells possess deficiency of HIF-1α expression, we still observed down-regulation of VEGF expression, suggesting that HIF-1 α is not the only one involved in this process.

COX-2 is also up-regulating VEGF in response to hypoxia and other stressors [15]. COX-2 and resultant prostaglandins promote tumor cell proliferation, resistance to apoptosis, and angiogenesis, in colorectal, oesophageal and lung cancer [16]. Other groups reported that COX-2 and PGE2 are key mediators of cellular responses observed after hypoxia and induction of VEGF [35, 36]. Our result show that COX-2 expression can be up regulated by hypoxia, however Rg3 decreased hypoxia stimulated overexpression of COX-2 in a dose-dependent manner in both cell lines. Furthermore, when COX-2 was decreased by celecoxib, a selective COX-2 inhibitor, subsequent reduction of VEGF expression was observed. These results indicate that COX-2 is also a pathway for inhibiting VEGF expression by Rg3.

Several studies have demonstrated that NF-κB regulates a variety of cellular processes, including angiogenesis, proliferation, invasion, migration, evasion of apoptosis, limitless replicative potential, to promote tumor development [37]. Compared with HIF-1α and COX-2, the regulation of NF-κB during hypoxia has been less studied. In resting cells, NF-κB is normally sequestered in the cytoplasm through its interaction with the IκB. After the phosphorylation and degradation of IκB, NF-κB is released and is free to translocate to the nucleus and to activate target genes [38, 39]. Although each of the family members of NF-κB (p65 (RelA), RelB, and c-Rel) can lead to gene transcription, the significant gene activation is triggered by the p65 expression [40]. We chose to examine whether Rg3 could inhibit p65 expression in Eca-109 and 786-0 cells under hypoxic condition. Our results showed that p65 expression was significantly inhibited when treated with Rg3 in both tumor cells.

Although the tumor cells in our study did have high levels of STAT3 and MAPKs activity, the hypoxia further elevated STAT3 and MAPKs activity by inducing their protein phosphorylation. Although STAT3 and MAPKs were required for hypoxia induced VEGF expression, STAT3 and MAPKs singal pathways have different mechanisms in the process of hypoxia-induced VEGF expression [22-26]. Our results about the role of STAT3 and MAPKs signaling in hypoxia-induced VEGF expression were consistent with other studies [22-26]. Moreover, STAT3, ERK1/2 and p38 protein phosphorylation induced by hypoxia were significantly restored by Rg3, but had no effect on JNK protein phosphorylation. Decreased protein phosphorylation led to decreased signaling activities. These results suggest that STAT3 and MAPKs signaling may play an important role in regulating VEGF expression in human cancer cells. The inhibitory effect of Rg3 on activities of STAT3, ERK1/2 and p38 signaling may also account for the decrease of VEGF expression.

Our results showed that HIF-1α, COX-2, NF-κB, STAT3 and MAPKs are all involved in the Rg3-induced VEGF suppression during hypoxia. Among these signaling pathways, HIF-1α seems to play the most critical role, as VEGF induction during hypoxia is mediated primarily through HIF-1α [30]. In addition to hypoxia, HIF-1α expression is regulated by many growth factors involving MAPK pathway [41]. In lung and colon cancer cells, HIF-1α is identified as a molecular link between the inflammatory and oncogenic pathways [42]. Interleukin-1β, a major inflammatory cytokine, up-regulates HIF-1α protein via an inflammatory signaling pathway involving NF-κB and COX-2, resulting in increased VEGF secretion in the lung and colon cancer cells [42]. Also in lung cancer cells, STAT3 is involved in the HIF-1α-induced upregulation of tumor gene expression [43]. In human renal cancer cells, hypoxia-activated STAT3 regulates the stability of HIF-1α protein and enhances HIF-1-mediated expression of VEGF [44]. Taken together, COX-2, NF-κB, STAT3 and MAPK are involved in the regulation of HIF-1α, and suppression of HIF-1α is likely the most important mechanism in the Rg3-induced VEGF suppression.

In conclusion, these experiments suggest that Rg3 may effectively suppress VEGF expression in cancer cells under normoxic and hypoxic conditions. These

actions may contribute to the overall efficacy of Rg3 against tumor angiogenesis and growth. The Rg3-induced VEGF suppression is related to the interruption of hypoxia-mediated and angiogenesis-related signaling pathways involving HIF-1α, COX-2, NF-κB, STAT3 and MAPKs. Further studies are required to elucidate the interplay of these signaling pathways in the Rg3-induced suppression of VEGF.

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