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Chaetoglobosin K inhibits tumor angiogenesis through downregulation of vascular epithelial growth factor-binding hypoxia-inducible factor 1α

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Abstract

Ovarian cancer is the fifth leading cause of cancer deaths for women in America. With no known carcinogens or manageable risk factors, targeted prevention is currently unavailable. Angioprevention is a nonspecific strategy to limit the growth of solid tumors and is especially suitable for ovarian cancers. In search of angiopreventive agents, we examined chaetoglobosin K (ChK), a natural cytochalasan compound from the fungus *Diplodia macrospora*. We found that ChK significantly inhibits cell viability at concentrations as low as 0.5 μmol/l for A2780/CP70 ovarian cancer cells and 1.0 μmol/l for OVCAR-3 cells. ChK also significantly inhibits the secretion of key angiogenesis mediators, including *Akt* (which is also known as protein kinase B), hypoxia-inducible factor 1α (*HIF-1α*), and vascular epithelial growth factor (*VEGF*) by ovarian cancer cells. More importantly, ChK inhibits in-vitro and in-vivo angiogenesis induced by ovarian cancer cells and reduces the migratory capability of human umbilical vein endothelial cells. Through transfection of *HIF-1α* plasmids in luciferase assays, we found that ChK executes its *VEGF* inhibition by mediating the downregulation of *HIF-1α*. Furthermore, chromatin immunoprecipitation assays using the *HIF-1α* antibody revealed that ChK inhibits the interaction of *HIF-1α* with the *VEGF* promoter. Through transfection of *Akt* plasmids, we found that inhibition of *HIF-1α* by ChK occurs through downregulation of *Akt*. To our knowledge, this is the first report about the potential angioprevention of ChK. Our data suggest that this natural fungal bioactive compound effectively inhibits angiogenesis through downregulation of *VEGF*-binding *HIF-1α* and could be an effective agent for cancer treatment.

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Conflicts of interest

There are no conflicts of interest.

Keywords

Akt; angiogenesis; chaetoglobosin K; hypoxia-inducible factor; ovarian cancer cells; vascular epithelial growth factor

Introduction

Despite an impressive reduction in death rates of several cancers in women over the past decades, including stomach, uterine, colon, rectal, and breast cancers, the death rates for ovarian cancers have remained unchanged [1]. Ovarian cancers are the fifth leading cause of cancer deaths among women [1], and recent studies have failed to provide an early diagnosis of ovarian cancers by means of screening biomarkers [2 – 4]. There are no known carcinogens or risk factors that can be avoided [5,6], and clinical treatment of ovarian cancers, mostly found at a late stage, offers limited hope in prognosis [7,8].

Meanwhile, all solid tumors growing beyond 1 mm in diameter face a limiting nutrition and oxygen supply and must establish their own blood vessel networks to support their own growth [9]. Angiogenesis, the establishment of new blood vessels [10], is therefore necessary for ovarian cancers to grow from early to late stages but is virtually quiescent in normal adult tissues [9]. Antiangiogenesis is one of the most promising strategies in the prevention and treatment of cancers [11] and is reported to be feasible for treating ovarian cancers [12].

In the search for antiangiogenic compounds for preventing and treating ovarian cancers, we recently screened over 20 bioactive natural compounds: genistein, kaempferol, apigenin, quercetin, tocopherol, tocopherol succinate, luteolin, rutin, naringin, taxifolin, paclitaxel, gallic acid, chaetoglobosin K (ChK), epicatechin, ellagic acid, catechin hydrate, baicalein, baicalin, tangeretin, nobiletin, and cisplatin for their effects on cell viability and on vascular epithelial growth factor (*VEGF*) secretion. Among the compounds screened, ChK had the strongest inhibition of cell viability and *VEGF* secretion in ovarian cancer cells. Therefore, in this study, we focused our anticancer research on ChK. The potential of bioactive compounds such as ChK from fungi has not been studied in much detail, although some flavonoids definitely showed effectiveness in anti-angiogenesis [10].

ChK is a bioactive compound isolated from *Diplodia macrospora*, a fungus responsible for ear rot and stalk rot of corn [13]. Although the structure of ChK was determined in 1980 [14], this compound remains largely unexplored, and only a few publications on ChK have been reported over the past three decades. These publications of in-vitro studies indicate that ChK suppresses the malignant phenotype of *ras*-transformed fibroblasts and induces apoptosis [15,16], prevents organochlorine-induced inhibition of gap junctional communication in astrocytes [17,18], inhibits Akt kinase phosphorylation and cytokinesis in *ras*-transformed liver epithelial cells [19], and plays a dual inhibition role in both *Akt* and *JNK* activation in *ras*-transformed epithelial and human lung carcinoma cells [20]. In this study, we examined the potentials of ChK in angioprevention of ovarian cancers and its underlying mechanisms. We assessed the effects of ChK on inhibiting the viability of, *VEGF* secretion in, in-vitro and in-vivo angiogenesis of, and human umbilical vein endothelial cell (HUVEC) migration of ovarian cancer cells. We hypothesized that ChK inhibits cancer angiogenesis through downregulation of hypoxia-inducible factor 1α (*HIF-1α*).

Materials and methods

Cell lines and reagents

ChK was dissolved in dimethyl sulfoxide (DMSO) as a 100 mmol/l stock solution and stored at – 80°C. The chemical structure of ChK is shown in Fig. 1. OVCAR-3 and A2780/CP70 ovarian cancer cell lines were obtained from Dr Binghua Jiang from West Virginia University [21]. Cancer cells were maintained in an RPMI-1640 medium (Sigma-Aldrich, St Louis, Missouri, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Grand Island, New York, USA). HUVEC cells were purchased from American Type Culture Collection (ATCC, Manassas, Virginia, USA) and maintained in a vascular cell basal medium (ATCC) supplemented with Endothelial Cell Growth Kit-VEGF (ATCC). All cells were cultured in a cell culture incubator with 5% CO_2 at 37°C.

Viability assay

OVCAR-3 and A2780/CP70 cells were seeded onto 96-well plates at 10 000 cells/well and incubated overnight before treatment with various concentrations of ChK (0.5, 1.0, 2.0, 4.0, 6.25, 12.5, and 25.0 μmol/l) and DMSO as a control for 24 h. Cell viability (reflecting the number of viable cells at the time period tested compared with the control) was analyzed with a CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, Wisconsin, USA), following the manufacturer's instructions, and normalized to control wells for statistical analysis. The experiments were conducted three times independently.

Enzyme-linked immunosorbent assay

VEGF protein secretion in ovarian cancer cells was evaluated using an enzyme-linked immunosorbent assay kit. A2780/CP70 and OVCAR-3 ovarian cancer cells were seeded at 10 000 cells/well onto 96-well plates and treated with 150 μl of RPMI-1640 medium containing ChK (0, 0.5, 1, and 2 μmol/l) for 24 h; 100 μl of culture supernatant was collected from each well for the *VEGF* assay, targeting $VEGF_{165}$ with a Quantikine Human *VEGF* Immunoassay Kit (R&D Systems, Minneapolis, Minnesota, USA) as per instructions. Standard curves generated from recombinant *VEGF* with known concentrations were used to quantify *VEGF* levels in the culture supernatants. The experiments were conducted three times.

In-vitro angiogenesis assay

A2780/CP70 and OVCAR-3 cancer cells were seeded onto six-well plates, incubated overnight, and treated with 2 ml of a serum-free medium containing ChK (0, 1, and 2 μmol/l) for 24 h. The conditioned medium was collected. For the in-vitro angiogenesis assay, growth factor-reduced Matrigels (BD Biosciences, San Jose, California, USA) were transferred into 96-well plates at a concentration of 50 μl/well and incubated at 37°C for 1 h to gel. HUVEC cells were harvested in PBS, counted, and seeded onto the Matrigel beds at 20 000 cells/90 μl PBS. Thereafter, volumes of 10 μl of the collected conditioned media were added to each well. The system was incubated at 37°C for 8 h, and photographed at × 50 amplification under a microscope. Each picture of 1388 × 1040 pixels was further divided by gridlines, and nine squares of 316 × 316 pixels were measured and averaged to obtain the tube length using the NIH ImageJ software (NIH, Bethesda, Maryland, USA) for statistical analysis.

In-vivo angiogenesis assay

All the experiments were performed on chicken embryos before hatching; our lab has been approved for using chicken embryos by the Institutional Animal Care and Use Committee, West Virginia University. Specific pathogen-free fertile chicken eggs (Charles River

Laboratories, North Franklin, Connecticut, USA) were incubated at 37.5°C and slowly turned by an automatic egg turner (G.Q.F. Manufacturing Company, Savannah, Georgia, USA). At day 7, the eggs were windowed to expose the chorioallantoic membrane (CAM). At day 9, A2780/CP70 cells (1.2×10^6 cells in a 20 μl FBS-free medium) were mixed with 80 μl Matrigel (BD Biosciences), treated with 2 μmol/l (final concentration) ChK or an equal concentration of DMSO freshly prepared in FBS-free medium, and implanted onto the CAM of chicken embryos. After incubating for another 5 days, tumor implants were photographed, and the number of blood vessels was calculated by two investigators blinded to the treatment. Scores from the two investigators were averaged for statistical analysis.

Migration assay

HUVEC cells were seeded onto six-well plates, cultured to 80% confluence, and stained for 30 min with 2 μmol/l calcein AM (BD Biosciences) freshly prepared in PBS. These cell monolayers were scraped with 10-μl pipet tips to generate two perpendicular acellular lines within each well, washed twice with PBS, and treated with ChK (0, 1, and 2 μmol/l) prepared in a vascular cell basal medium supplemented with Endothelial Cell Growth Kit-VEGF for 8 h. At 0 and 8 h, the acellular lines within each well were photographed at ×50 amplification under a fluorescence microscope (485 nm excitation/520 nm emission) and counted for the total number of cells that migrated to the acellular areas.

Western blotting

A2780/CP70 and OVCAR-3 ovarian cancer cells (1.5×10^6/dish) were seeded onto 60-mm dishes and incubated overnight. Cells were then treated with various concentrations (0, 0.5, 1, and 2 μmol/l) of ChK for 2 h for *Akt* determination or for 24 h for detection of other proteins. Cells were lysed with M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, Illinois, USA) supplemented with Halt Protease and Phosphatase Inhibitor (Pierce) as per instructions. Cell lysates were separated by SDS-PAGE gel electrophoresis and immunoblotted with antibodies against phospho-*Akt*, total *Akt* (Cell Signaling Technology, Danvers, Massachusetts, USA), *HIF-1α* (BD Biosciences), phospho-mTOR, mTOR (R&D Systems), *PTEN* and *GAPDH* (Santa Cruz Biotechnology, Santa Cruz, California, USA), *NF-κB*, and *c-Myc*. Protein bands were visualized using SuperSignal West Pico substrate (Pierce) and X-ray films (Phenix Research, Candler, North Carolina, USA), quantified with the NIH ImageJ software, and normalized to corresponding load-control proteins for statistical analysis. The experiments were conducted three times.

Transient transfection and reporter assay

Ovarian cancer cells (10 000 cells/well) were seeded onto 96-well plates and incubated overnight. For transfection with *HIF-1α* plasmids (Addgene, Cambridge, Massachusetts, USA), cells were transfected with *VEGF* luciferase reporter (0.01 γg), β-galactosidase (0.01 γg), and *HIF-1α* (0, 0.02, 0.04, and 0.08 γg) or SR-α (as vehicle) plasmids using 0.2 μl of jetPRIME reagent (VWR, West Chester, Pennsylvania, USA) for 4 h, followed by a 16-h treatment with or without ChK (0 and 2 μmol/l). The cells were harvested and analyzed for luciferase activity using a ONE-Glo Luciferase Assay System (Promega) and for β-galactosidase activity using a β-Galactosidase Enzyme Assay System (Promega), as previously described [22]. The levels of *VEGF* reporter were normalized to corresponding β-galactosidase activities. For transfection with m*Akt* plasmids (Addgene), cells were transfected with *VEGF* luciferase reporter (0.05 μg) and with mAkt (0, 0.05, 0.1, and 0.2 μg) or SR-α plasmids using 1 μl of lipofectamine2000 reagent (Invitrogen) for 5 h, followed by an 8-h treatment with or without ChK (0 and 2 μmol/l). The cells were harvested and analyzed for luciferase activity and total protein levels using a BCA Protein Assay Kit (Pierce), and the activities of *VEGF* reporter were normalized to corresponding total protein levels for statistical analysis. The experiments were conducted three times.

Chromatin immunoprecipitation assay

A2780/CP70 cells (7.5×10^6 cells/dish) were seeded onto 100-mm dishes and incubated overnight. Cells were then treated with or without ChK (0 and 2 μmol/l) for 24 h. After treatment, the chromatin immunoprecipitation (ChIP) assay was performed using an EZ ChIP kit (Millipore, Temecula, California, USA) as per the manufacturer's instructions. In brief, cross-linked cells were collected, lysed, sonicated, and subjected to immunoprecipitation with the *HIF-1α* antibody (Millipore). Immunocomplexes were collected using protein A/G agarose beads and eluted. Cross-links were reversed by incubating the samples at 65°C. DNA was extracted and purified. The DNA sequences for the primers used to amplify the *VEGF* promoter were 5′-AGACTCCACAGTGCATACG TG-3′ (forward) and 5′-AGTGTGTCCCTCTGACAA TG-3′ (reverse).

Samples were analyzed by real-time PCR using the Chromo4 real-time detector (Bio-Rad, Hercules, California, USA). The cycle threshold (C_t) values for each sample were normalized to the C_t values of the input samples to obtain ΔC_t. The differences between the normalized experimental samples and the control samples were determined to obtain $\Delta\Delta C_t$. The normalized fold difference relative to the control sample was then calculated using $2^{-\Delta\Delta C_t}$. Three independent experiments were carried out.

Statistical analysis

Data from three independent experiments were combined for statistical analysis and presented as means±SE. One-way analysis of variance, followed by Dunnett's test or a *t*-test, was performed, as appropriate, to determine the differences between the groups using the SPSS software (SPSS Inc., Armonk, New York, USA). A *P*-value of less than 0.05 was considered significant.

Results

ChK reduces the viability of ovarian cancer cells

The effects of ChK on the viability of A2780/CP70 and OVCAR-3 ovarian cancer cells were first evaluated and optimized. We found that, compared with controls (ChK 0 μmol/l), ChK significantly reduced the viability of A2780/CP70 cells at all concentrations (0.5–25.0 μmol/l) studied and also significantly reduced the viability of OVCAR-3 cells at ChK concentrations of 1.0 μmol/l and above (Fig. 2). At 25.0 μmol/l, no viable cells were detected. At the same concentration, no significant differences were observed between A2780/CP70 and OVCAR-3 cells. The results showed that ChK is potent in reducing the viability of ovarian cancer cells.

ChK inhibits *VEGF* secretion in ovarian cancer cells

For the purpose of angioprevention, levels of *VEGF*, an important growth factor, were examined. We found that ChK significantly reduced *VEGF* secretion by A2780/CP70 cells at concentrations of 1.0 and 2.0 μmol/l and by OVCAR-3 cells at 0.5, 1.0, and 2.0 μmol/l (Fig. 3). At the same ChK concentration, no significant differences were observed between A2780/CP70 and OVCAR-3 cells.

ChK inhibits in-vitro angiogenesis induced by ovarian cancer cells

Reduced secretion of *VEGF* (Fig. 3) by ovarian cancer cells treated with ChK is expected to inhibit angiogenesis because of VEGF's pivotal role in angiogenesis [10]; therefore, we tested in-vitro tube formation by HUVEC cells induced by the culture medium of ovarian cancer cells treated with different concentrations of ChK. We found that the culture media conditioned by both A2780/CP70 and OVCAR-3 ovarian cancer cells promoted in-vitro

angiogenesis and presented a well-established network of HUVEC cells, and the medium from A2780/CP70 cells seemed to be more effective in promoting in-vitro angiogenesis compared with the medium from OVCAR-3 cells (ChK = 0 μmol/l, Fig. 4a). ChK treatment, however, led to truncated HUVEC networks (ChK = 1 and 2 μmol/l, Fig. 4a). A significant reduction in tube length was observed at ChK concentrations of 1 and 2 μmol/l for A2780/CP70 cells and at 2 μmol/l for OVCAR-3 cells when compared with corresponding controls (Fig. 4b).

ChK inhibits in-vivo angiogenesis induced by ovarian cancer cells

To test the hypothesis that effective inhibition of *VEGF* secretion by ChK treatment can be translated into antiangiogenic effects *in vivo*, CAM assays using chicken embryos were performed to determine the effect of ChK treatment on ovarian cancer angiogenesis *in vivo*. As shown in Fig. 5, ChK treatment at concentrations of 2 μmol/l significantly reduced the generation of new blood vessels that was induced by A2780/CP70 ovarian cancer cells.

ChK inhibits the migration of HUVEC cells in plastic culture vessels

HUVEC cell migration assays were performed to determine whether ChK affects the migration of HUVEC cells in plastic culture vessels, as this is relevant to ChK's antiangiogenic attributes. We found that after introducing a ‘wound’ by scraping monolayer HUVEC cells in culture vessels, the ‘wound’ showed signs of ‘healing’ 8 h later by means of HUVEC cells migrating into the acellular area (ChK = 0 μmol/l, Fig. 6). However, ChK treatment impaired the migratory capabilities of HUVEC cells cultured in plastic vessels, and at a ChK concentration of 2 μmol/l, significantly fewer HUVEC cells migrated into the acellular areas when compared with controls (Fig. 6).

ChK inhibits *VEGF* secretion by regulating *HIF-1* expression and the interaction of *HIF-1α* with the *VEGF* promoter

Because of the close regulation of *VEGF* secretion by the *HIF-1α* gene, the expression of *HIF-1α* at the protein level was determined. ChK treatment significantly inhibited *HIF-1α* protein expression in both A2780/CP70 and OVCAR-3 cells at concentrations of 0.5, 1, and 2 μmol/l (Fig. 7a and b).

To prove that *HIF-1α* expression is not only regulated by ChK treatment but also plays a role in ChK's inhibition on *VEGF* secretion, A2780/CP70 and OVCAR-3 cells were transfected with the *VEGF*-promoter reporter, together with *HIF-1α* plasmids. Although ChK (2 μmol/l) treatment significantly inhibited *VEGF* transcriptional activation, this inhibition was significantly reversed by forced expression of the *HIF-1α* protein, and the higher the expression of *HIF-1α*, the higher the expression of the *VEGF* reporter (Fig. 7c and d).

To examine whether ChK was involved in targeting *HIF-1α* to the promoter of *VEGF*, ChIP assays with *HIF-1α* antibody were performed, followed by real-time PCR using primers for the *VEGF* promoter. Results showed that the ChK (2 μmol/l) treatment of A2780/CP70 cells significantly inhibited the interaction of *HIF-1α* with the *VEGF* promoter and resulted in significantly less *VEGF* secretion when compared with controls (Fig. 7e).

ChK inhibits *VEGF* secretion through *Akt/mTOR* signaling

The phosphorylation of *Akt* was significantly inhibited by ChK treatment at concentrations of 0.5, 1, and 2 μmol/l when compared with controls (Fig. 8a and b). After transfecting with the *VEGF*-promoter reporter and m*Akt* plasmids, *VEGF* transcriptional activation was significantly reduced by ChK (2 μmol/l) treatment in both A2780/CP70 and OVCAR-3 cells, whereas the effect was significantly reversed by forced expression of the *Akt* protein

at a concentration of 0.2 μg of the m*Akt* plasmid (Fig. 8c and d). The phosphorylation of *mTOR* was significantly inhibited by ChK treatment at a concentration of 2 μmol/l when compared with controls (Fig. 8e and f).

We also looked into protein expressions of *PTEN*, *NF-κB*, and *c-Myc* genes in A2780/CP70 and OVCAR-3 cells treated with and without ChK (0, 0.5, 1, and 2 μmol/l). No significant differences were found among the concentrations studied in both A2780/CP70 and OVCAR-3 cells (Fig. 8g and h).

Discussion

A transcription factor controls and regulates gene expression by binding to a particular promoter or enhancer. HIF-1α is a key transcription factor that has been reported to regulate over 258 different genes [23]. HIF-1α is an important mediator of angiogenesis and thus holds great promise as a therapeutic target of angiogenesis [21,24]. This study reported the functional effects of the cytochalasan compound ChK obtained from *D. macrospora* as a potent regulator of *HIF-1α*.

It is generally thought that *HIF-1α* is regulated mainly by low oxygen or hypoxia as its name indicates. However, we found that *HIF-1α* is highly expressed in normoxic conditions in ovarian cancer cells [21]. Recent evidence indicates that several nonhypoxic factors can also mediate *HIF-1α* expression, such as natural nonpeptide bioactive compounds, cytokines, and oncogenic factors [21,24], suggesting that ChK may be such a factor for regulating *HIF-1α* under nonhypoxic conditions. *HIF-1α* inhibition has not been reported for cytochalasan compounds to our knowledge. Therefore, there is a need to explore ChK as a novel inhibitor of angiogenesis in cancer prevention and treatment.

This study indicates that ChK is an effective *HIF-1α* protein inhibitor and antiangiogenesis agent. In general, the *PTEN/PI3K/Akt/mTOR* pathway plays an important role in regulating protein synthesis [25,26]. We found that ChK inhibits Akt phosphorylation in ovarian cancer cells in the *PTEN/PI3K/Akt/mTOR* pathway. Our finding agrees well with the finding that ChK inhibited *Akt* phosphorylation in *ras*-transformed epithelial cells [19]. These results suggest the involvement of the PI3K pathway in the inhibition of *VEGF* secretion and angiogenesis. We also found that ChK did not affect protein expression of *PTEN*, *NF-κB*, and *c-Myc* genes in our ovarian cancer system.

The mechanism by which ChK inhibits the *PI3K/Akt* signaling pathway is not yet fully understood; however, it has been shown that ChK inhibited *Akt* phosphorylation and cytokinesis in *ras*-transformed epithelial cells [19]. In human lung carcinoma and *ras*-transformed epithelial cells, ChK inhibits *JNK* and *Akt* phosphorylation [20]. The implication of the bioactive cytochalasan compound inhibiting *HIF-1α* is intriguing as *HIF-1α* has been shown to be required in *VEGF* secretion, which is essential for tumor angiogenesis [21]. Through ChIP assays using the *HIF-1α* antibody, we found that ChK inhibits the interaction of *HIF-1α* with the *VEGF* promoter. By transfecting *HIF-1α* plasmids in luciferase assays, we found that ChK executes its *VEGF* inhibition by mediating the hypoxia-independent downregulation of *HIF-1α*. Through transfection of *Akt* plasmids, we found that inhibition of HIF-1α by ChK is through inhibition of *Akt* phosphorylation.

We found that ChK significantly inhibited cell viability in both A2780/CP70 and OVCAR-3 ovarian cancer cells. ChK treatment significantly reduced *VEGF* secretion in the two ovarian cancer cell lines. Consistent with our hypothesis, ChK treatment effectively reduced angiogenesis induced by ovarian cancer cells *in vitro* and *in vivo* and suppressed the migration of HUVEC cells cultured in plastic vessels in the HUVEC cell migration assay. ChK was found to inhibit *VEGF* secretion in ovarian cancer cells through the *Akt–HIF–*

VEGF pathway (Fig. 9). The potency exhibited at low ChK concentrations studied may indicate that ChK is an effective antiangiogenesis agent.

Conclusion

This study revealed an important mechanism of the antiangiogenic activity of ChK and found that ChK inhibits tumor angiogenesis through downregulation of *HIF-1α*. ChK was found to significantly inhibit cell viability and *VEGF* protein secretion in two ovarian cancer cells (i.e. A2780/CP70 and OVCAR-3). ChK was also found to effectively reduce angiogenesis *in vitro* and *in vivo* and HUVEC cell migration. In addition, ChK was found to inhibit *VEGF* secretion in ovarian cancer cells through the *Akt–HIF–VEGF* pathway. This is the first report on angiogenesis inhibition through downregulation of *VEGF*-binding *HIF-1α* by the nonpeptide cytochalasan compound ChK in cancer cells. These findings enhance our understanding of its working mechanism and provide strong support for using this compound in animal model studies.

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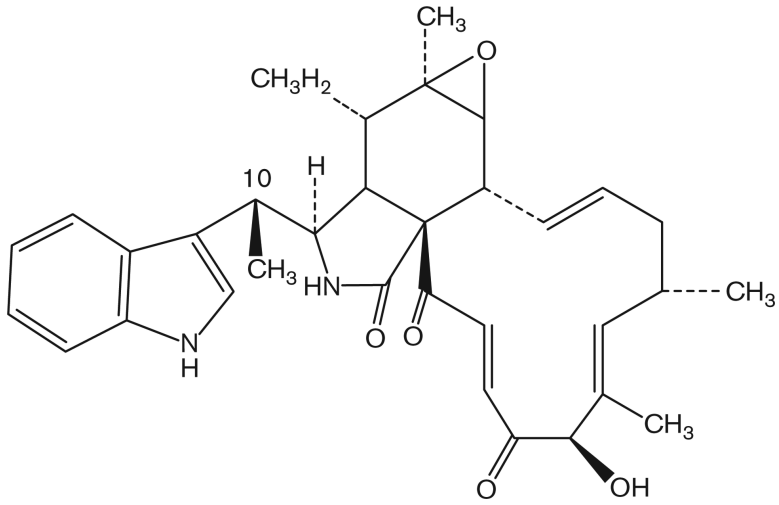

Fig. 1.
The chemical structure of chaetoglobosin K (ChK).

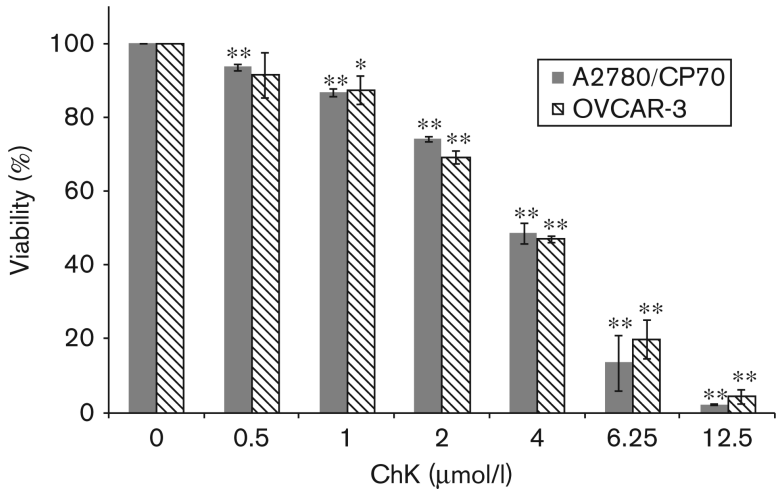

Fig. 2.

ChK inhibits the viability of ovarian cancer cells. A2780/CP70 and OVCAR-3 cells were seeded and incubated overnight before treatment with ChK (0–12.5 μmol/l) for 24 h. *$P <$ 0.05 compared with controls. **$P <$ 0.01 compared with control. ChK, chaetoglobosin K.

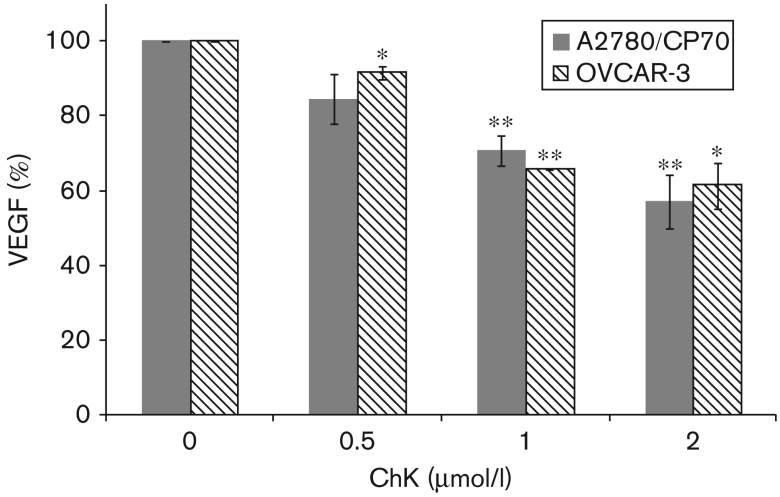

Fig. 3.

ChK inhibits VEGF protein secretion in ovarian cancer cells. A2780/CP70 and OVCAR-3 cells were seeded and incubated overnight before treatment with ChK (0, 0.5, 1, and 2 μmol/l) for 24 h. *$P < 0.05$ compared with controls. **$P < 0.01$ compared with controls. ChK, chaetoglobosin K; VEGF, vascular epithelial growth factor.

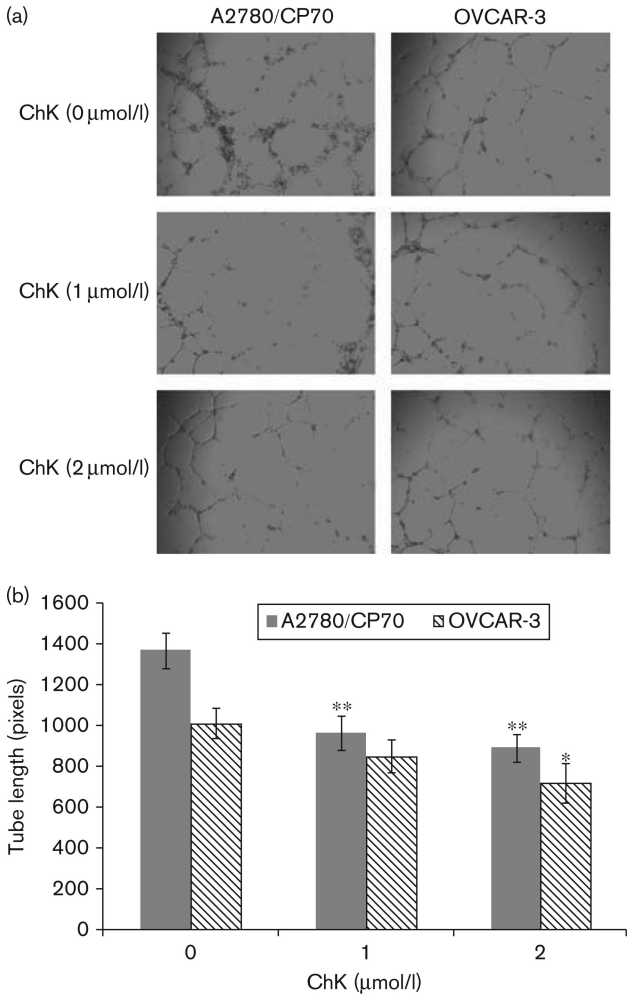

Fig. 4.

ChK inhibits in-vitro angiogenesis induced by ovarian cancer cells. A2780/CP70 and OVCAR-3 cells were seeded, treated with ChK (0, 1, and 2 μmol/l) for 24 h, and the media were collected. Growth factor-reduced Matrigels were aliquoted and incubated at 37°C for 1 h to gel. HUVEC cells were harvested, counted, and seeded onto the gelled Matrigel beds. The collected conditioned cell culture media were then added to each well. The system was incubated at 37°C for 8 h. (a) Photographs of the in-vitro angiogenesis assay. (b) Tube lengths of A2780/CP70 and OVCAR-3 cells treated with and without ChK. $*P < 0.05$ compared with controls. $**P < 0.01$ compared with controls. ChK, chaetoglobosin K; HUVEC, human umbilical vein endothelial cell.

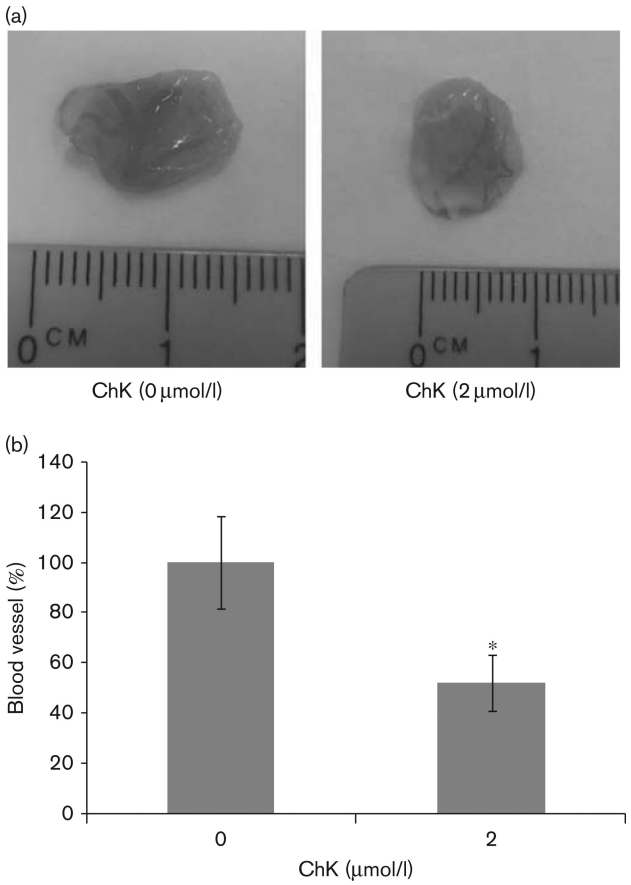

Fig. 5.

ChK inhibits in-vivo angiogenesis induced by A2780/CP70 ovarian cancer cells. A2780/CP70 cells were mixed with Matrigel, treated with ChK (0 and 2 μmol/l), and implanted onto the chorioallantoic membrane of 9-day-old chicken embryos. After incubating for 5 days, tumor implants were photographed, and the number of blood vessels was evaluated. (a) Typical tumor implants treated with and without ChK (0 and 2 μmol/l). (b) Blood vessels of tumor implants treated with and without ChK (0 and 2 μmol/l). *$P < 0.05$ compared with controls. ChK, chaetoglobosin K.

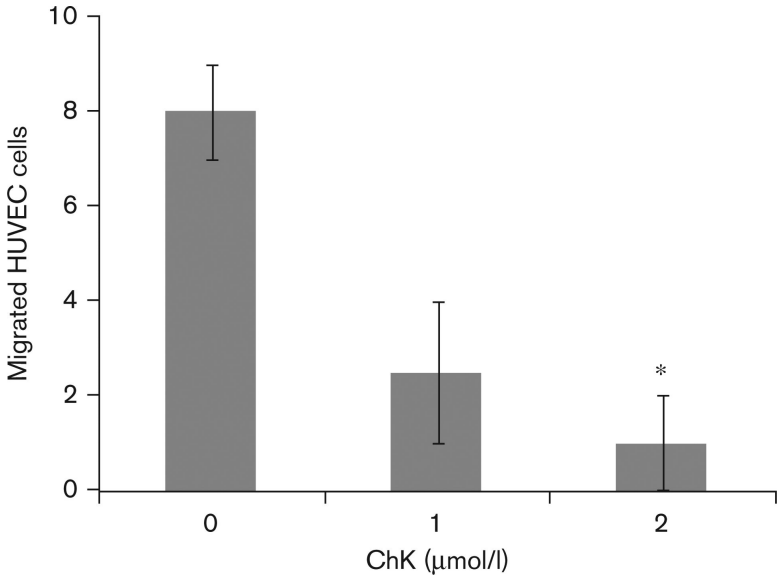

Fig. 6.

ChK inhibits the migration of HUVEC cells. HUVEC cells were seeded, cultured to confluence, and stained with calcein AM. The cells were scraped to make two perpendicular acellular lines within each well, washed, and then treated with media containing ChK (0, 1, and 2 μmol/l) for 8 h. At 0 and 8 h, the acellular lines within each well were photographed and counted for the number of migrated cells. *$P < 0.05$ compared with controls. ChK, chaetoglobosin K; HUVEC, human umbilical vein endothelial cell.

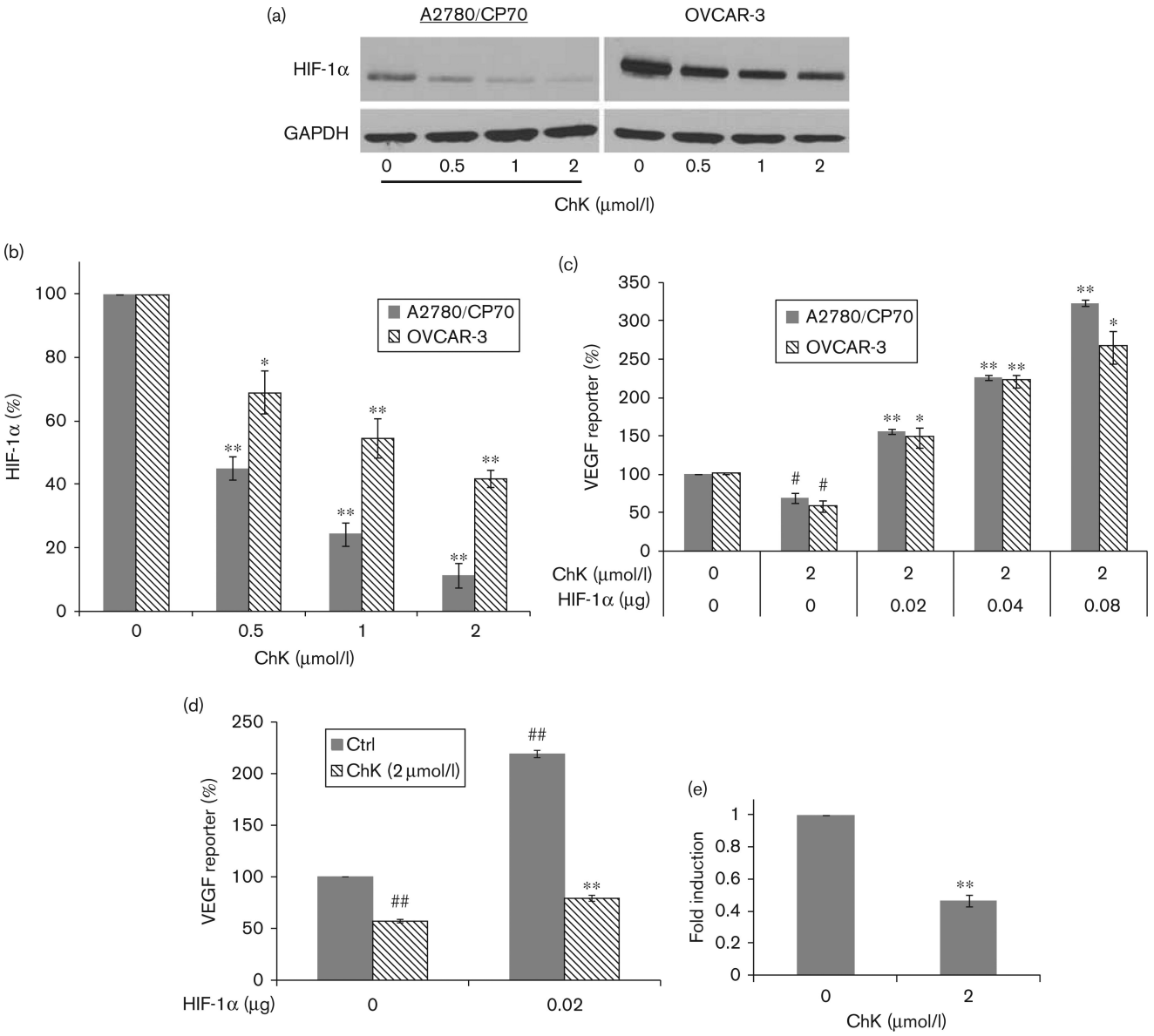

Fig. 7.

ChK regulates VEGF secretion in ovarian cancer cells by inhibiting HIF-1α gene expression. (a) Western blotting pictures of HIF-1α and (b) bar graph of the western blotting analysis of A2780/CP70 and OVCAR-3 cells. A2780/CP70 and OVCAR-3 cells (1.5×10^6 cells/dish) were seeded onto 60-mm dishes, incubated overnight, and treated with ChK (0, 0.5, 1, and 2 μmol/l) for 24 h. Cells were harvested and analyzed by SDS-PAGE and western blotting. $*P < 0.05$ compared with controls. $**P < 0.01$ compared with controls. (c) Normalized VEGF reporter levels. A2780/CP70 and OVCAR-3 cells (1.0×10^4 cells/well) were seeded onto 96-well plates and incubated overnight. Cells were then transfected for 4 h, followed by a 16-h treatment with or without ChK (0 and 2 μmol/l). $\#P < 0.05$ compared with controls. $*P < 0.05$ compared with ChK (2 μmol/l)-treated controls. $**P < 0.01$ compared with ChK (2 μmol/l)-treated controls. (d) Normalized VEGF reporter levels. A2780/CP70 cells (1.0×10^4 cells/well) were seeded onto 96-well plates and incubated overnight. Cells were then transfected with HIF-1α for 4 h, followed by a 16-h treatment with or without ChK (0 and 2 μmol/l). $\#\#P < 0.01$ compared with controls at HIF-1α = 0 μg.

**$P < 0.01$ compared with controls at HIF-1α = 0.02 μg. (e) Fold induction of VEGF secretion. A2780/CP70 cells (7.5×10^6 cells/dish) were seeded onto 100-mm dishes, incubated overnight, and treated with or without ChK (0 and 2 μmol/l) for 24 h. After treatment, the chromatin immunoprecipitation assay and PCR analysis for the VEGF gene promoter were performed. **$P < 0.01$ compared with controls. ChK, chaetoglobosin K; Ctrl, control; HIF-1α, hypoxia-inducible factor 1α; VEGF, vascular epithelial growth factor.

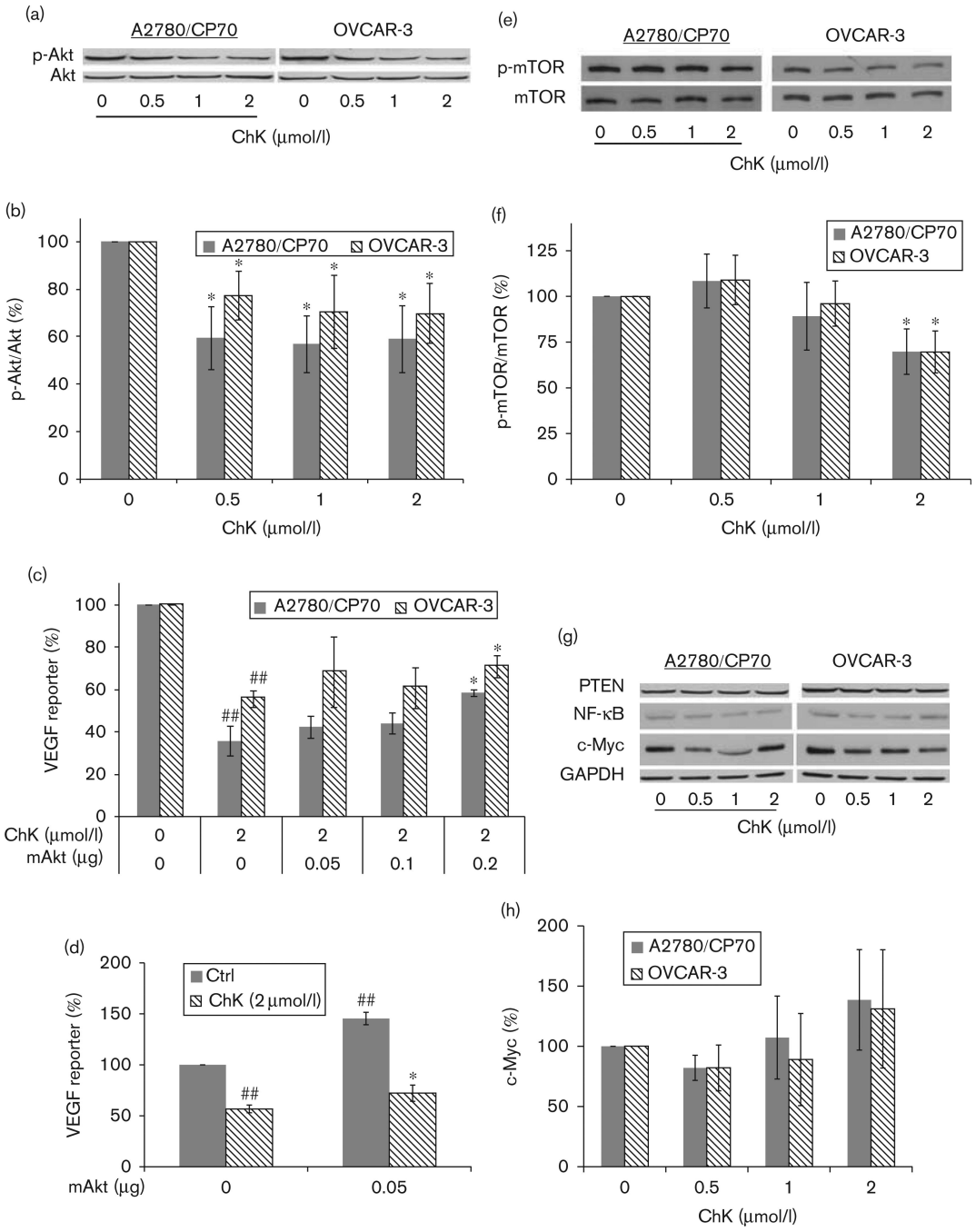

Fig. 8.

ChK regulates the phosphorylation of Akt and mTOR but not the expression of PTEN in ovarian cancer cells. (a) Western blot images of Akt and (b) bar graph of the western blot analysis of A2780/CP70 and OVCAR-3 cells. A2780/CP70 and OVCAR-3 cells (1.5×10^6 cells/dish) were seeded onto 60-mm dishes, incubated overnight, and treated with ChK (0, 0.5, 1, and 2 μmol/l) for 2 h. Cells were harvested and analyzed using SDS-PAGE and western blotting. p-Akt protein levels were normalized by total Akt protein bands and expressed as percentages of the control. $*P < 0.05$ compared with controls. (c) Normalized VEGF reporter levels. A2780/CP70 and OVCAR-3 cells were seeded onto 96-well plates and incubated overnight. Cells were then transfected with VEGF luciferase reporter and

mAkt or SR-α plasmids using the lipofectamine2000 reagent, followed by treatment with or without ChK (0 and 2 μmol/l). ##$P < 0.01$ compared with control. *$P < 0.05$ compared with ChK (2 μmol/l)-treated control. (d) Normalized VEGF reporter levels of A2780/CP70 cells. A2780/CP70 cells (1.0×10^4 cells/well) were seeded onto 96-well plates and incubated overnight. Cells were then transfected with the mAKT plasmid for 4 h, followed by a 16-h treatment with or without ChK (0 and 2 μmol/l). ##$P < 0.01$ compared with controls at mAkt = 0 μg. *$P < 0.05$ compared with controls at mAkt = 0.05 μg. (e) Western blot images of mTOR and (f) bar graph of the western blot analysis of A2780/CP70 and OVCAR-3 cells. A2780/CP70 and OVCAR-3 cells (1.5×10^6 cells/dish) were seeded onto 60-mm dishes, incubated overnight, and treated with ChK (0, 0.5, 1, and 2 μmol/l) for 2 h. Cells were harvested and analyzed using SDS-PAGE and western blotting. p-mTOR protein levels were normalized by total mTOR protein bands and expressed as percentages of the control. *$P < 0.05$ compared with controls. (g) Effect of ChK on protein expressions of PTEN, NF-κB, and c-Myc, and GAPDH. A2780/CP70 and OVCAR-3 cells (2×10^6 cells/dish) were seeded onto 60-mm dishes, incubated overnight, and treated with ChK (0, 0.5, 1, and 2 μmol/l) for 24 h. Cells were harvested and analyzed using SDS-PAGE and western blotting. (h) Bar graph of c-Myc protein levels. ChK, chaetoglobosin K; Ctrl, control; VEGF, vascular epithelial growth factor.

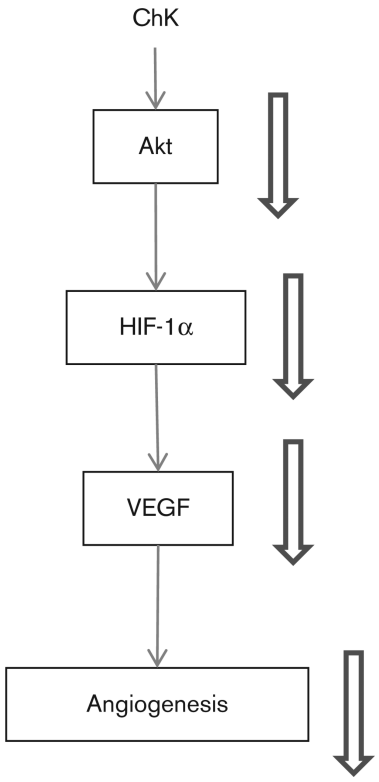

Fig. 9.
The proposed pathway for ChK's effects on in-vivo angiogenesis. ChK, chaetoglobosin K; HIF-1α, hypoxia-inducible factor 1α; VEGF, vascular epithelial growth factor.