Chrysin inhibits expression of hypoxia-inducible factor-1α through reducing hypoxia-inducible factor-1α stability and inhibiting its protein synthesis

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Abstract

Chrysin is a natural flavonoid and has been shown recently to have anticancer effects. However, the mechanisms that chrysin inhibits cancers are not well known. In this study, we investigated the effects of chrysin on expression of hypoxia-inducible factor-1α (HIF-1α) and vascular endothelial growth factor in human prostate cancer DU145 cells. Chrysin inhibited insulin-induced expression of HIF-1α by reducing its stability. Chrysin increases ubiquitination and degradation of HIF-1α by increasing its prolyl hydroxylation. In addition, chrysin interfered with interaction between HIF-1α and heat shock protein 90. Chrysin was also found to inhibit HIF-1α expression through AKT signaling. Inhibition of HIF-1α by chrysin resulted in abrogation of vascular endothelial growth factor expression. Finally, we showed that chrysin inhibited DU145 xenograft-induced angiogenesis in nude mice. Taken together, these results suggest that chrysin is a potent inhibitor of HIF-1α and provide a new sight into the mechanisms of chrysin against cancers. [Mol Cancer Ther 2007;6(1):220–6]

Introduction

Flavonoids are present in fruits, vegetables, and beverages derived from plants and in many dietary supplements or herbal remedies (1). Flavonoids have been described as health-promoting, disease-preventing dietary supplements and have activity as cancer-preventive agents (1). Additionally, they are extremely safe and associated with low toxicity, making them excellent candidates for chemopreventive agents. Flavonoids comprise several classes, including flavones, flavanones, flavanols, and flavans. Chrysin (5,7-dihydroxyflavone) is a natural flavonoid. Several studies in recent years have shown that chrysin has multiple biological activities, such as anti-inflammation, anticancer, and antioxidation effects (2–4). However, the molecular mechanisms underlying chrysin anticancer effects are not well understood.

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcriptional factor composed of α and β subunits. HIF-1 is overexpressed in many human cancers (5), and the levels of its activity in cells correlate with tumorigenicity and angiogenesis (6). HIF-1α is induced by hypoxia, growth factors, and oncogenes (7, 8). HIF-1α is constitutively expressed but rapidly degraded by the ubiquitin-proteasome pathway under normoxia (9, 10). The prolyl hydroxylation of HIF-1α at the oxygen-dependent degradation domain (ODD) is critical in the regulation of HIF-1α steady-state levels (11–14). Under hypoxic condition, the absence of oxygen prevents the prolyl hydroxylase from modifying HIF-1α, allowing HIF-1α to accumulate (11, 12). Vascular endothelial growth factor (VEGF) is mainly regulated by HIF-1α at transcriptional level (15). VEGF plays a critical role in tumor angiogenesis (16). Angiogenesis is the formation of new blood vessels from preexisting ones and is required for tumor growth and metastasis (17). Therefore, an antiangiogenic therapy that targets the HIF-1α/VEGF system is a promising strategy for the treatment of cancers.

Prostate cancer is the second leading cause of cancer-related death in men, behind lung cancer in Western countries (18). HIF-1α and VEGF are overexpressed in prostate cancer cells (19–22). Angiogenesis plays an important role in prostate cancer (23). Insulin stimulates the proliferation of prostate cancer cell line *in vitro* (24), and elevated level of insulin is a risk factor for the development of prostate cancer and for recurrence of prostate cancer in patients following radiation treatment (25, 26). It was found that insulin induced expression of HIF-1α and VEGF in prostate cancer cells (27). The discovery of a new agent that targets HIF-1α and VEGF is a potentially effective chemotherapeutic treatment for human prostate cancer. In this study, we showed that chrysin inhibited insulin-induced expression of HIF-1α and VEGF in human prostate cancer DU145 cells via multiple pathways. Chrysin inhibited DU145 xenograft-induced angiogenesis *in vivo*. Our results provide a new insight into the mechanisms of the inhibitory effects of chrysin on cancers.

Materials and Methods

Materials

Chrysin was purchased from Sigma (St. Louis, MO), and its purity was >96%. Antibodies against HIF-1α and HIF-1β were from BD Biosciences (Franklin Lakes, NJ). Protein A/G plus-agarose and antibody against green fluorescent protein (GFP) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody against heat shock protein 90 (Hsp90) was purchased from BD Biosciences (San Jose, CA). The antibodies against AKT and phosphorylated AKT were from Cell Signaling (Beverly, MA). The proteasome inhibitor MG132 and protein synthesis inhibitor cycloheximide were from Sigma. The phenol red–free Matrigel is a product of BD Biosciences (Bedford, MA).

Cell Culture

DU145 cells and human renal epithelial 293T cells were maintained in RMPI 1640 and DMEM, respectively. All media contained 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin. Cells were grown in 5% CO_2, 37°C. Cell culture under hypoxia was done as described previously (28).

Immunoblotting, Immunoprecipitation, and ELISA Assay

Western blotting was done as described (28). For immunoprecipitation, 500 μg of cell lysates were incubated with 1 μg of Hsp90 antibody in the presence of 30 μmol/L of chrysin at 4°C; 500 μg of cell lysates incubated with equal volume of solvent alone under the same conditions were used as control. In 3 h, 20 μL of Protein A/G plus-agarose beads was added, and the incubation was continued. In 6 h, the beads were washed at least four times. Finally, 30 μL of SDS-PAGE loading buffer (2×) was added, and the sample was heated at 100°C for 3 min. The immunoprecipitates were resolved in 8% SDS-PAGE. Hsp90 was used as loading control. VEGF protein was determined by ELISA as described previously (28).

Construction of Plasmids

VEGF promoter reporter pMAP11wt and plasmid encoding human HIF-1α were constructed as described (15, 29). Active myr-AKT was constructed as described (28). The pCMV-ODD-GFP vector (encoding ODD-GFP) and pCMV-GFP are kind gifts from Dr. D'Angelo (Institut de Pharmacologie Moleculaire et Cellulaire du Centre National de la Recherche Scientifique, France; ref. 30). The ODD fragment corresponds to amino acids 530 to 603 of HIF-1α. Based on ODD-GFP, the pCMV-ODD(P564A)-GFP was prepared by PCR. The primers used are sense, GGAGATGTTAGCTGCCTATATCCCAATGG and antisense, CCATTGGGATATAGGCAGCTAACATCTCC. This construct encodes a mutant ODD that the proline residue at 564 was replaced with alanine. This mutation will prevent ODD from hydroxylation by prolyl hydroxylase.

Transient Transfection and Luciferase Assay

293T cells were transfected using calcium phosphate method. DU145 cells were transfected using LipofectAMINE from Invitrogen (Carlsbad, CA; ref. 28). Luciferase activities were determined as described (28). Relative luciferase activity (defined as VEGF reporter activity) was calculated as the ratio of luciferase/β-galactoside activity.

In vivo Angiogenesis Assay

Tumor angiogenesis was done as described previously (31). In brief, DU145 cells were mixed with Matrigel at a ratio of 1:2 (v/v, %), in the presence or absence of chrysin (30 μmol/L); 0.45 mL of the mixture (total cell number = 3×10^6) was injected s.c. into the flank sides of BALB/c-nu nude mice (male, 4 weeks old, purchased from Shanghai Experimental Animal Center, Shanghai, China). In 11 days, the Matrigel plugs were removed from the mice and trimmed of the surrounding tissues. Hemoglobin content of the gel plug was measured as described (31). The data represented the mean ± SD ($n = 8$) from two independent experiments.

### Statistics Analysis

The data represent mean ± SD from three independent experiments, except where indicated. Statistical analysis was done by Student's t test at a significance level of $P < 0.05$.

Results

Chrysin Inhibits HIF-1α Expression in DU145 Cells

Under serum-free condition, protein level of HIF-1α was low in DU145 cells (Fig. 1A). Insulin induced HIF-1α expression, which was inhibited by chrysin in a dose-dependent manner (Fig. 1A). We next determined the kinetics that chrysin inhibits insulin-induced HIF-1α. As shown in Fig. 1B, insulin induced HIF-1α expression to the maximum in 6 h. Within the time tested, chrysin inhibited HIF-1α expression significantly (Fig. 1B). Chrysin did not affect expression of HIF-1β (Fig. 1). Thus, in our next experiments, we chose HIF-1β as loading control.
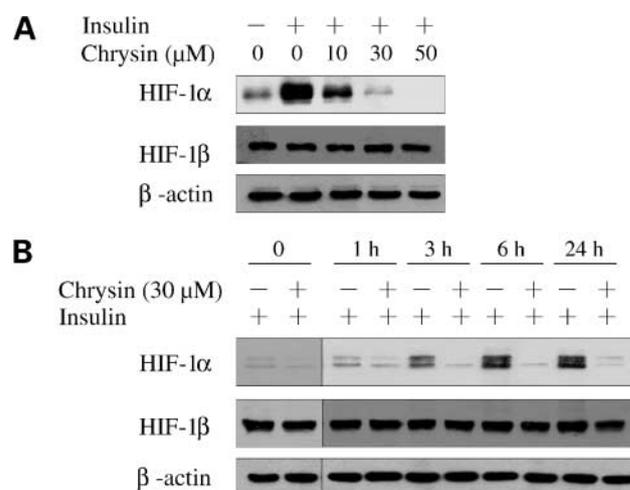
Figure 1. Chrysin inhibited insulin-induced HIF-1α. **A,** DU145 cells were cultured to 60% to 70% confluence and switched to serum-free medium. In 24 h, the cells were pretreated with different dose of chrysin (0, 10, 30, and 50 μmol/L) for 30 min. Insulin (200 nmol/L) was added, and the cells were incubated for 6 h. The cells were harvested, lysed, and immunoblotted as described in Materials and Methods. **B,** serum-starved DU145 cells were pretreated with chrysin for 30 min, followed by treatment with insulin (200 nmol/L) for different times as indicated.
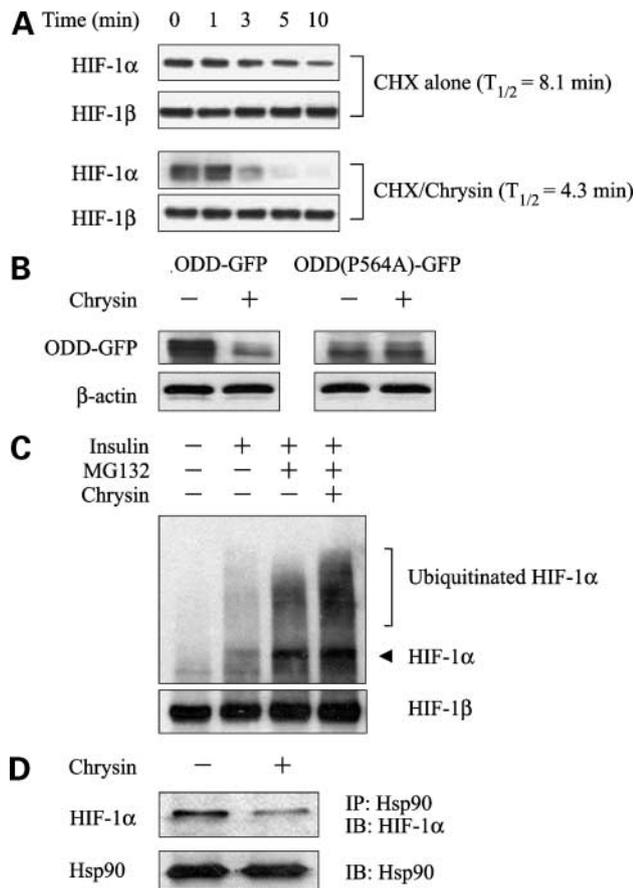
Figure 2. Chrysin reduces HIF-1α stability. **A,** chrysin reduced the half-life of HIF-1α. Serum-starved DU145 cells were stimulated with insulin (200 nmol/L) for 6 h, followed by treatment with 30 μmol/L chrysin for 30 min. Cycloheximide (*CHX*; 100 μg/mL) was added, and the cells were harvested at different times. Levels of HIF-1α protein were determined by measuring the density of the HIF-1α band and normalizing to that of HIF-1β. Experiments were done three times. **B,** chrysin promoted degradation of ODD-GFP. 293T cells were transfected with ODD-GFP or ODD(P564A)-GFP as described in Materials and Methods. In 36 h, the transfected cells were treated with chrysin (30 μmol/L) for 6 h. ODD-GFP was determined by immunoblotting using antibody against GFP. **C,** chrysin increased ubiquitination of HIF-1α. The serum-starved DU145 cells were stimulated with insulin (200 nmol/L) for 6 h. MG132 (20 μmol/L) or MG132 plus chrysin (30 μmol/L) was added, and the cells were treated for 1 h. The cells were harvested, and cell lysates were prepared for immunoblotting. **D,** chrysin interfered with interaction between HIF-1α and Hsp90. DU145 cells were grown in serum-containing medium to 80% to 90% confluence. The cells were harvested, and cellular proteins were prepared for immunoprecipitation as describe in Materials and Methods.

Chrysin Inhibits HIF-1α Expression via Reducing Its Stability

The half-life of HIF-1α protein was ~8.1 min (Fig. 2A). If the cells were treated with chrysin, the half-life of HIF-1α was reduced to 4.3 min (Fig. 2A). These results suggest that chrysin inhibits expression of HIF-1α by reducing its stability. To know the possible mechanism, we tested the effects of chrysin on ODD. As shown in Fig. 2B, chrysin inhibited expression of ODD-GFP but had no effects on that of ODD(P564A)-GFP. These results suggest that chrysin promotes degradation of HIF-1α through enhancing the

prolyl hydroxylation of ODD of HIF-1α. Prolyl hydroxylation of ODD facilitates ubiquitination and thereafter proteasome degradation of HIF-1α. We found that chrysin further increased ubiquitinated HIF-1α by MG132 (Fig. 2C), suggesting that chrysin increases prolyl hydroxylation of ODD and promotes ubiquitination and thereafter degradation of HIF-1α.

HIF-1α belongs to the Per-ARNT-Sim basic helix-loop-helix family and interacts with Hsp90 (32). Binding of HIF-1α with Hsp90 prevents HIF-1α from degradation (33, 34). We found that chrysin inhibited binding of HIF-1α to Hsp90 (Fig. 2D), suggesting that chrysin promotes HIF-1α degradation via interfering with the interaction between Hsp90 and HIF-1α.
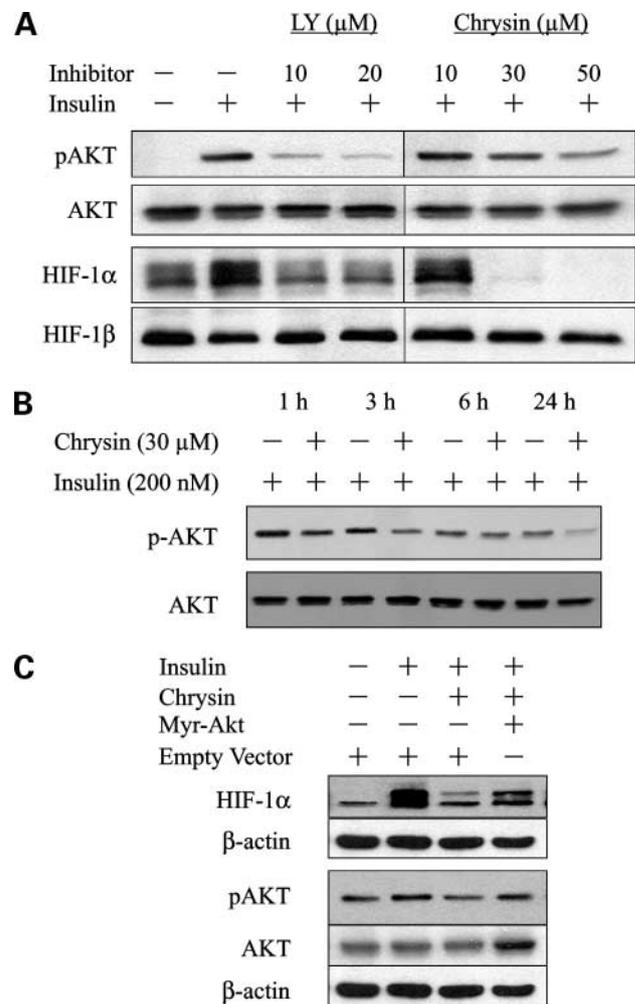
Figure 3. Chrysin inhibits expression of HIF-1α through AKT signaling. **A,** serum-starved DU145 cells were pretreated with LY294002 or chrysin for 30 min, followed by stimulation with insulin (200 nmol/L) for 6 h. LY, LY294002. **B,** serum-starved DU145 cells were pretreated with chrysin for 30 min, followed by addition of insulin. The cells were incubated for different times as indicated. **C,** DU145 cells in 60-mm dish were transfected with active myr-AKT (4 μg) or empty vector (4 μg). In 24 h, the cells were switched to serum-free medium and incubated overnight. Then, the starved DU145 cells were pretreated with chrysin (30 μmol/L) for 30 min, followed by induction with insulin (200 nmol/L) for 6 h.

Chrysin Regulates HIF-1α Expression through the Phosphatidylinositol 3-Kinase/AKT Pathway

It has been reported that AKT signaling plays a role in HIF-1α expression (27, 35). Insulin activated AKT and induced HIF-1α expression (Fig. 3A). LY294002, the specific inhibitor of phosphatidylinositol 3-kinase, attenuated insulin-induced phosphorylation of AKT and expression of HIF-1α. Similarly, chrysin inhibited insulin-induced AKT phosphorylation and HIF-1α expression (Fig. 3A). We also determined the kinetics of chrysin on AKT phosphorylation. AKT phosphorylation was inhibited by chrysin within the time tested (Fig. 3B). Overexpression of myr-AKT reversed chrysin-inhibited HIF-1α expression (Fig. 3C). These results suggest that chrysin inhibits HIF-1α expression through AKT signaling.

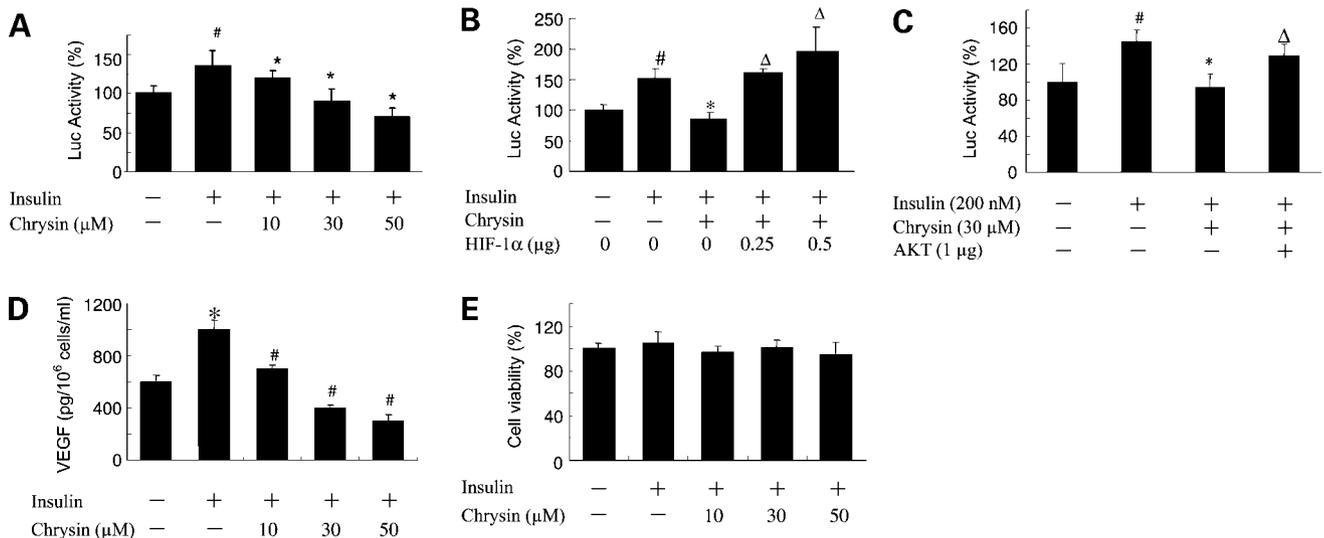### Chrysin Inhibits VEGF Transcriptional Activation and VEGF Expression

To know whether or not chrysin inhibits expression of VEGF, we first determined the effects of chrysin on VEGF transcriptional activation in DU145 cells. Insulin increased VEGF reporter activity (Fig. 4A). Pretreatment of the cells with chrysin abrogated insulin-induced VEGF reporter activity in a dose-dependent manner (Fig. 4A). Overexpression of HIF-1α reversed the chrysin-inhibited VEGF reporter activity (Fig. 4B). These results suggest that chrysin inhibits VEGF transcriptional activation through

HIF-1α. In addition, we found that overexpression of active AKT reversed the chrysin-inhibited VEGF transcriptional activation (Fig. 4C), suggesting that chrysin suppresses VEGF expression via AKT signaling.

In our subsequent experiments, we determined the effects of chrysin on production of VEGF protein. As shown in Fig. 4D, chrysin inhibited insulin-induced expression of VEGF in DU145 cells. To exclude the possibility that the inhibition of VEGF formation was due to cell death by chrysin, we determined the cell viability by trypan blue staining as described previously (36). Under the same experimental conditions, chrysin had little effects on viability of DU145 cells (Fig. 4E).

Chrysin Affects HIF-1α and VEGF Expression under Hypoxic Condition

Effect of chrysin on HIF-1α was determined under hypoxia in DU145. Hypoxia induced expression of HIF-1α, which was blocked by chrysin (Fig. 5A). The effect of chrysin on VEGF production under hypoxia was also examined. Hypoxia induced VEGF expression, and treatment of cells with chrysin decreased the VEGF production (Fig. 5B). Chrysin had little effects on DU145 viability under the same experimental conditions (data not shown). Taken together, these results suggest that chrysin inhibits expression of HIF-1α and VEGF under both hypoxic and normoxic conditions.

Figure 4. Chrysin inhibits VEGF expression. **A,** chrysin attenuated VEGF transcriptional activation. DU145 cells were transfected with VEGF promoter reporter and β-galactoside plasmid as described (28). The transfected cells were cultured overnight and then switched to serum-free medium, and the incubation was continued for 20 h. The starved cells were pretreated with chrysin (0, 10, 30, and 50 μmol/L) for 30 min, followed by stimulation with insulin (200 nmol/L) for 15 h. #, $P < 0.05$ versus control; *, $P < 0.05$ versus the group treated with insulin. **B,** chrysin inhibits VEGF transcriptional activation through HIF-1α. DU145 cells in 12-well plates were transfected with VEGF reporter plasmid, β-galactoside plasmid, and HIF-1α (0, 0.25, and 0.5 μg) plasmid. Empty vector was added to adjust to the same amounts of plasmids transfected. In 24 h, the transfected cells were switched to serum-free medium. In 20 h, the starved cells were pretreated with chrysin (30 μmol/L) for 30 min followed by addition of insulin (200 nmol/L). The cells were incubated for 15 h. #, $P < 0.05$ versus control; *, $P < 0.05$ versus the group treated with insulin alone; Δ, $P < 0.05$ versus the group of insulin plus chrysin. **C,** overexpression of myr-AKT reversed chrysin-inhibited VEGF transcriptional activation. The experiment was done as described in (**B**), except that the HIF-1α plasmid was replaced by the myr-AKT vector. #, $P < 0.05$ versus control; *, $P < 0.05$ versus the group treated with insulin alone; Δ, $P < 0.05$ versus the group of insulin plus chrysin. **D,** chrysin inhibited insulin-induced expression of VEGF. Serum-starved DU145 cells were pretreated with chrysin for 30 min; 200 nmol/L insulin was added, and the cells were incubated for 18 h. *, $P < 0.05$ versus the starved cells; #, $P < 0.05$ versus insulin-treated cells. **E,** DU145 cells viability under the conditions of (**D**) was determined as described in Materials and Methods.
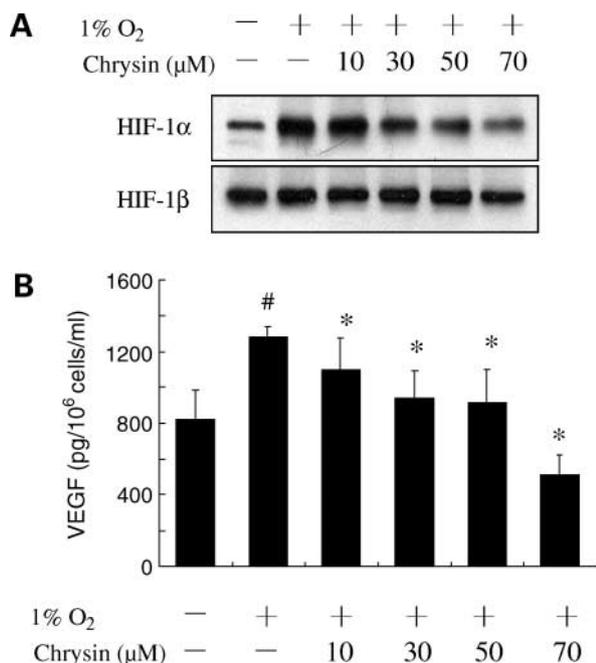
Figure 5. Chrysin inhibits HIF-1α and VEGF expression under hypoxic condition. **A,** DU145 cells were pretreated with chrysin for 1 h and then exposed to 1% oxygen for 6 h. **B,** DU145 cells were cultured to ~90% confluence. Chrysin was added, and the cells were incubated under hypoxia (1% O_2) for 18 h. VEGF in the medium was measured by ELISA. The results were normalized to the cell number and medium volume. *, $P < 0.05$ versus solvent-treated cells; #, $P < 0.05$ versus cells under hypoxia.

Chrysin Inhibits *In vivo* Tumor Angiogenesis

In vivo Matrigel plug assay is frequently used to determine antiangiogenic effects of drugs and reagents. To test whether chrysin inhibits tumor angiogenesis *in vivo*, DU145 cells were mixed with Matrigel in the presence or absence of chrysin and injected into the flank of the male nude mice as described in Materials and Methods. The mice were sacrificed, and the gel plugs were harvested 11 days after injection. Hemoglobin content of the xenograft was determined as an index of angiogenesis. Matrigel alone did not induce angiogenesis (Fig. 6A and B). However, the DU145 xenografts induced angiogenesis obviously (Fig. 6A and B). Treatment of DU145 cells with chrysin inhibited significantly the xenograft angiogenesis (Fig. 6A and B).

Discussion

Epidemiologic studies have suggested that the dietary intake of fruits and vegetables can reduce incidences of many types of cancer (37–39). Dietary factors contribute to about one third of potentially preventable cancers, and the preventive effects of plant-based diets on tumorigenesis and other chronic diseases have been well documented (40). Several cancers, including prostate cancer, have a lower incidence in Asia than in Western countries. This has been attributed to the Asian dietary regimen, which is typically rich in flavonoid-containing plants. Chrysin, a

common flavonoid, has been proposed as an antitumor agent (41–43). However, the molecular mechanisms involved are not well understood. In this article, we have shown that chrysin impaired VEGF expression via HIF-1α in human prostate cancer DU145 cells.

Chrysin reduced the half-life of HIF-1α (Fig. 2A) and increased the degradation of ODD-GFP but not that of ODD(P564A)-GFP (Fig. 2B), suggesting that chrysin reduces HIF-1α stability via increasing prolyl hydroxylation of ODD. Increase of prolyl hydroxylation of ODD will result in increase of ubiquitination and proteasome degradation of HIF-1α. This is confirmed by results of Fig. 2C. Hsp90, a chaperone protein, has been shown to stabilize HIF-1α (34, 44). Chrysin was found to inhibit the binding of HIF-1α to Hsp90, suggesting that chrysin inhibits HIF-1α expression via interfering with the interaction between HIF-1α and Hsp90 (Fig. 2D).

Expression of HIF-1α is regulated not through protein degradation but also through protein synthesis. For example, it has been reported that insulin stimulated HIF-1α expression through phosphatidylinositol 3-kinase and target of rapamycin signaling (45). Phosphatidylinositol 3-kinase/AKT signal pathway plays an important role in the expression of HIF-1α (27, 35). We found that chrysin inhibited phosphorylation of AKT in DU145 cells (Fig. 3A and B), and overexpression of active AKT reversed the chrysin-inhibited HIF-1α expression (Fig. 3C). These results suggest that chrysin inhibits HIF-1α protein synthesis through AKT signaling.

To our knowledge, it is the first time to show that flavonoid chrysin inhibits HIF-1α via prolyl hydroxylation
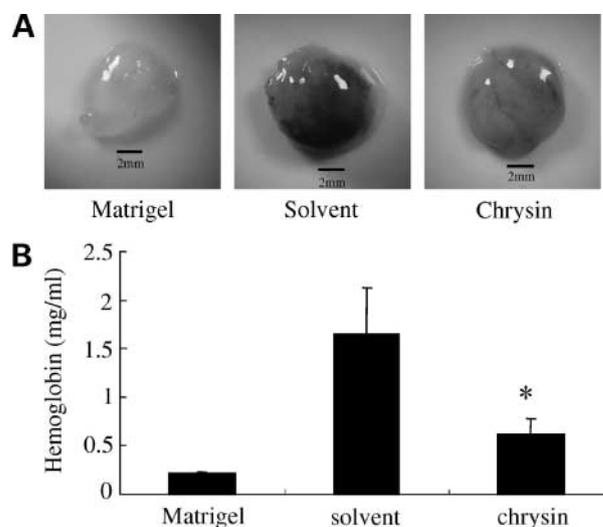
Figure 6. Chrysin inhibits DU145 xenograft-induced angiogenesis. DU145 cells were mixed with Matrigel and injected into the flank of male nude mice as described in Materials and Methods. In 11 days, the mice were euthanized, and the Matrigel plugs were removed and photographed immediately. **A,** representative gel plugs. Bar, 2 mm. **B,** measurement of hemoglobin contents of the gel plugs. Relative hemoglobin content is the hemoglobin level (mg) divided by the volume of each plug (mL). *, $P < 0.05$ versus cells treated with solvent alone.

of ODD. It will be interesting to know the mechanisms of how chrysin affects prolyl hydroxylation of ODD. For example, does chrysin inhibit prolyl hydroxylase activity directly? Many flavonoids were found inhibitor of phosphatidylinositol 3-kinase/AKT signaling. Chrysin inhibited AKT activation (Fig. 3). Thus, it is conceivable that chrysin may also inhibit HIF-1α via AKT signaling. It was reported that flavonoids or related compounds, such as apigenin (28, 46), resveratrol (47), and (−)-epigallocatechin-3-gallate (48), inhibited expression of HIF-1α through multiple pathways, including protein synthesis and protein degradation. Consistent with previous reports, we show here that chrysin is also multifunctioning in HIF-1α pathway. This might be due to the nonspecific binding of these compounds to cellular molecules. It must be noted that all these studies were done at cell level *in vitro*. Some biological effects of the compound observed *in vitro* may not occur *in vivo* because the concentration used *in vitro* is usually high. Thus, it would be interesting to know which are the more important or relevant mechanisms *in vivo*.

We showed here that chrysin suppressed expression of HIF-1α of tumor cells *in vitro* and inhibited tumor cell–induced angiogenesis *in vivo*. Because HIF-1α regulates not only VEGF but also a number of other genes that are related to cancer (49), it is likely that chrysin can be a potent inhibitor of angiogenesis and tumorigenesis.

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