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ARTICLE

Chrysin Suppresses IL-6-Induced Angiogenesis via Down-regulation of JAK1/STAT3 and VEGF: An in Vitro and in Ovo Approach

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Chrysin, 5,7-dihydroxyflavone, possesses many biologic properties. This study aimed to investigate the effects and molecular mechanisms of chrysin on IL-6-induced angiogenesis in vitro and in ovo. Chicken chorioallantoic membrane assay, an in ovo angiogenesis assay, showed chrysin significantly suppressed IL-6-induced neovascularization. Furthermore, chrysin significantly suppressed human umbilical vein endothelial cell (HUVECs) migration and tube formation. The signaling pathway involved in chrysin-related antiangiogenesis was also investigated. The data indicated that chrysin is able to down-regulate the expression of glycoprotein 130 (gp130), soluble IL-6 receptor (IL-6R), phosphorylated JAK1 and STAT3, and VEGF in HUVECs. The IL-6-induced binding of STAT3 was significantly suppressed by chrysin. Moreover, chrysin did not further suppress VEGF expression with STAT3 knocked down. Taken together, the results show that chrysin suppresses IL-6-induced angiogenesis through modulation of the sIL-6R/gp130/JAK1/STAT3/VEGF signaling pathway. Chrysin may provide new therapeutic potential for IL-6-induced pathological angiogenesis.

KEYWORDS: IL-6; chrysin; angiogenesis; VEGF; gp130/JAK1/STAT3

INTRODUCTION

Chrysin (5,7-dihydroxyflavone) is one of the natural flavonoids derived from *Scutellaria baicalensis* Georgi (**Figure 1**). It has been shown to possess several biological activities, including anticancer (*1*, *2*). The antiangiogenic activity of food-derived polyphenols as potent inhibitors of tumor growth has increased (*2*). However, few studies have evaluated its effect on angiogenesis and the regulation of molecular mechanisms of angiogenic factors in endothelial cells, including the chrysin-mediated suppression of interleukin 6 (IL-6)-induced VEGF (*3*).

The development and discovery of new agents with antiangiogenic effects would be key for managing these over-angiogenic diseases. In the process of neovasculization, the quiescent endothelial cells respond to stimulation such as inflammation and hypoxia (*4*, *5*). Once the stimulation is activated, endothelial cells escape from the stable vascular basement membrane and secrete angiogenic factors. Accumulating evidence indicates that IL-6 is an important prognostic factor in angiogenic pathological disorders, such as coronary artery diseases and many cancers (*6*, *7*). Recent investigations have documented the relationship between IL-6 and VEGF in cardiovascular diseases and cancers (*8*, *9*). The interplay of IL-6 and VEGF is associated with the transduction of membrane glycoprotein 130 (gp130) and the subsequent intracellular activation of Janus kinase 1/signal transducer and activator of transcription (JAK/STAT) phosphorylation in endothelial cells (*10*). Furthermore, activation of STAT3 leads to the up-regulation of VEGF and tumor angiogenesis. Chen and his colleagues have reported that the blockage of STAT3 signaling in tumors can reduce tumor angiogenesis (*11*).

In this study, we investigated the effects and molecular mechanisms of chrysin on IL-6-induced angiogenesis, including its modulation of the interplay between IL-6R/JAK1/STAT3 in human umbilical vein endothelial cells (HUVECs).

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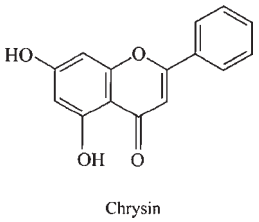

Chrysin

Figure 1. Structure of chrysin.

METHODS AND MATERIALS

HUVEC Culture. HUVECs were isolated from human umbilical veins. After flushing with cold phosphate-buffered saline (PBS), the HUVECs were isolated as described previously (*12*).

Chemical Reagents. High-purity chrysin (>98%) was purchased from Wako Pure Chemical Industry (Osaka, Japan). Endothelial cell-based medium (EBM) was purchased from Cambrex Bio Science, Inc. (Walkersville, MD), and Medium 199 was purchased from GIBCO BRL (Grand island, NY). IL-6 was obtained from CytoLab Ltd. (Rehovot, Israel), and trysolol (aprotinin) was purchased from Bayer CropScience Co. Ltd. (Leverkusen, Germany). Dimethyl sulfoxide (DMSO), penicillin, and streptomycin were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). DMSO (1 ppm) was added to each vehicle group in these experiments. Matrigel basement membrane matrix was acquired from Becton Dickinson (Bedford, MA). Antibodies were purchased from R&D Systems (Minneapolis, MN) or Cell Signaling Technology, Inc. (Beverly, MA).

Chorioallantoic Membrane (CAM) Assay. The CAM assay was used for neovascularization tests in ovo (*13*). The study protocol was performed with minor modification to the protocol previously described (*14*). Blood vessels were counted and photographed with a Nikon digital camera. The photograph was evaluated and quantified under the processing of the PhotoImpact 8.0 (Ulead, Taiwan) software. Six eggs were included in each independent experiment. The assay was performed six times. Each sample was normalized to eggs incubated without stimulation to determine the ratio of new vessel formations.

Cell Migration Assay and Tube Formation Assay. The cell migration assay and tube formation assay were applied to examine the phenotypic alterations of HUVECs. The HUVEC migration assay followed the modified method of Liu's staining protocol mentioned previously (*14*, *15*). All experiments were performed in triplicate, and each provided similar results.

Quantification of VEGF, sIL-6R, gp130, T- and P-JAK1, and STAT3 Proteins. Western blot analysis was performed as previously described (*16*). VEGF, sIL-6R, gp130, total and phosphorated-JAK1, and STAT3 antibodies were purchased from R&D Systems. Each experiment was done in triplicate, and each sample was normalized to cells incubated without stimulation to determine the ratio of protein expression.

Electrophoretic Mobility Shift Assay (EMSA). An electrophoretic mobility shift assay was performed as described previously (*17*). Oligonucleotide sequences included the STAT3 binding region consensus (5′-GAT CCT TCT GGG AAT TCC TAG ATC-3′) and mutant (5′-GAT CCT TCT GGG ccg TCC TAG ATC-3′) forms. After annealing, these double-stranded oligonucleotides were labeled with (γ-^{32}P) ATP by T4 polynucleotide kinase (Mission Biotech Co., Taipei, Taiwan) as probes. Controls were performed in each case with mutant oligonucleotides competing with the labeled sequence. The binding reaction contained 20000 cpm of DNA probe, 1 μg of poly(dIdC), 25 mM HEPES (pH 7.9), 10 mM KCl, 0.2 mM EDTA, 0.2 mM dithiothreitol, 10% glycerol, 5 mM $MgCl_2$, 0.45 M NaCl, and 5 μg of nuclear proteins collected from different conditions. Protein−DNA complex formation was carried out on ice for 20 min and resolved by a 5% nondenaturing polyacrylamide gel using 0.5 μg of Tris borate−EDTA buffer (44.5 mM Tris-base, 44.5 mM boric acid, 1 mM EDTA, pH 8.0) at 4 °C. Gels were dried, and bands were detected using autoradiographic film. Each assay was done in triplicate.

Transfection of Small Interfering STAT3 RNA into HUVECs. HUVECs were seeded into wells at a density of 10^6 cells per well in 2% EBM endothelial cell culture medium. All of the cells were incubated for 24 h under CO_2 at 37 °C. After incubation, the HUVECs were transfected with STAT3 small interfering RNA (siRNA) (siGENOME SMARTpool; Dharmacon, Chicago, IL) (800 ng of siRNA in 5 mL of culture medium) or nontargeting siRNA (siCONTROL Nontargeting RNA, Dharmacon) using LipofectAMINE2000. Four pairs of 21-bp RNA duplexes with a TT overhang at the 3′ end that specifically targets STAT3 mRNA were purchased (siGEMONE SMARTpool, Dharmacon). A 27-mer RNA duplex that specifically targets STAT3 mRNA was designed and synthesized (IDTDNA, Caralville, IA): sense, 5′-CCA CUU UGG UGU UUC AUA AUU-3′; and antisense, 5′-UAA UGA AAC ACC AAA GUG GUU-3′. GFP siRNA (sense, 5′-UUC UCC GAA CGU GUC ACG UdTdT-3′; and antisense, 5′-ACG UGA CAC GUU CGG AGA AdTdT-3) was used as a control. After incubation, the transfection medium was removed and replaced with antibiotic-free EBM medium. Inhibition of STAT3 was verified by quantitative real-time PCR and Western blotting analysis. Protein expression of VEGF (downstream of STAT3) was examined after introduction of the siRNA for STAT3.

Statistical Analysis. All data in different experimental groups are expressed as means ± SD. Statistical significance was evaluated by analysis of variance (ANOVA). The Tukey−Kramer comparison test was used for pairwise comparisons between multiple groups after the ANOVA. Bonferroni's test, the Wilcoxon signed rank, or the rank sum test was used to compare multiple groups to a single control group. A p value of < 0.05 was considered to be statistically significant.

RESULTS

Inhibitory Effect of Chrysin on Neovascularization in Ovo. According to the MTT assay, the half-maximal inhibitory concentration (IC_{50}) of chrysin on endothelial cell cultures was 7.7×10^{-4} M (data not shown). Chrysin was therefore studied at a concentration range of 10^{-8}−10^{-5} M in the following experiments. The VEGF protein expression was increased in a time course manner by IL-6 induction in HUVEC cultures, and the VEGF protein level revealed a plateau pattern after 12 h of IL-6 induction (data not shown). A CAM assay was performed to evaluate the antiangiogenic effect of chrysin in ovo. New vessel formation in HUVECs was assessed after IL-6 (10 ng/mL) induction with or without chrysin (10^{-8}−10^{-5} M). There was a statistically significant increase in neovascularization after IL-6 induction, and secondary and tertiary vascular formations were significantly attenuated in chrysin-treated cells in a concentration-dependent manner (**Figure 2**).

Effects of Chrysin on Phenotypic Alterations of HUVECs Induced by IL-6. To evaluate the effects of chrysin on phenotypic alterations of HUVECs induced by IL-6, a cell migration assay and tube formation assay were performed. The migration of endothelial cells and the formation of capillary-like, branched vessel networks occurred within 18 h after IL-6 treatment (10 ng/mL). Chrysin significantly suppressed IL-6-induced cellular migration (**Figure 3A**,**C**) and new vessel and branched network formation (**Figure 3B**,**C**). The dedicated information of counting endothelial cell migration, new vessel, and branched and network formation is referenced from Lin's work (*14*).

Inhibitory Effects of Chrysin on sIL-6R/gp130 and JAK1/STAT3 Expressions. We further investigated the possible antiangiogenesis mechanisms of chrysin through the IL-6 transmembrane and intracellular signaling pathway, including sIL-6R and gp130, which form complexes with IL-6 for its rapid internalization and action (*10*). Treatment with chrysin resulted in the attenuation of protein expression of both IL-6R and gp130 in a concentration-dependent manner (**Figure 4A**). Moreover, treatment with chrysin (10^{-8}−10^{-5} M) definitely decreased the levels of the phosphorylated form of both JAK1 (phosphorylation at Tyr1022/1023) and STAT3 proteins (phosphorylation at Tyr705) in HUVECs induced by IL-6 for 12 h (**Figure 4B**).

Effects of Chrysin on the Interaction of STAT3 and VEGF. STAT3 is the most important transcription factor in the IL-6 transmembrane and intracellular signaling pathway (*17*).

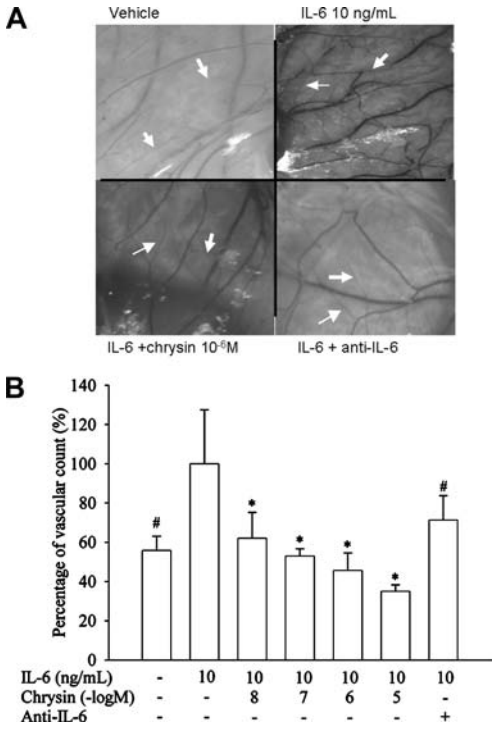

Figure 2. Antiangiogenic effects of chrysin and quantification of neovascularization on chicken chorioallantoic membrane (CAM). New vessel formations in CAMs after induction by 10 ng/mL IL-6, combined with either chrysin (10^{-6} M) or neutralized IL-6 antibody (5 μg/mL, positive control), were evaluated as described under Materials and Methods. Data are presented as the percentages of the control group, which was set at 100%. The arrow indicated new vessel formation from main vascular branch. $*$, $p < 0.05$ versus IL-6-treated group (one-way ANOVA); #, $p < 0.05$ versus IL-6-treated group (Wilcoxon rank sum test).

We further investigated the potential role of STAT3 in antiangiogenic effects of chrysin. First, an EMSA was performed to test the binding activity of STAT3. Interestingly, chrysin reversed this induction in a concentration-dependent manner (10^{-8}–10^{-5} M) (**Figure 5A**). Second, we further studied the regulation of VEGF by chrysin via STAT3 by siRNA knockdown. Either chrysin or STAT3 knockdown significantly attenuated IL-6-induced VEGF protein (**Figure 5B**). However, simultaneous administration of chrysin plus STAT3 siRNA did not show a higher suppression effect of VEGF than either chrysin or STAT3 siRNA. These results imply that chrysin suppresses IL-6-induced VEGF expression by suppressing STAT3.

DISCUSSION

Interleukin-6 is one of the important inducers of angiogenesis, which contributes to the process of many human diseases (*5*). Increasing amounts of evidence have revealed that flavonoids are enriched with antiangiogenic activities and show important angioprotection against many human diseases, especially tumor growth (*14*, *18*). Chrysin, one kind of common flavonoid, has been shown to be a nutrient with anticancer and anti-inflammatory effects (*2*), although its mechanisms of activity are not clear. Our study shows that IL-6 induces human endothelial cell activation by inducing the IL-6/IL-6R and JAK/STAT3 pathways, which leads to the induction of VEGF. Furthermore, our study is the first to report that chrysin suppresses IL-6-induced angiogenesis in ovo and in vitro and that the effect is due to the inhibition of the IL-6 receptor intracellular signaling pathway IL-6R/gp130/JAK1/STAT3/VEGF in human endothelial cells.

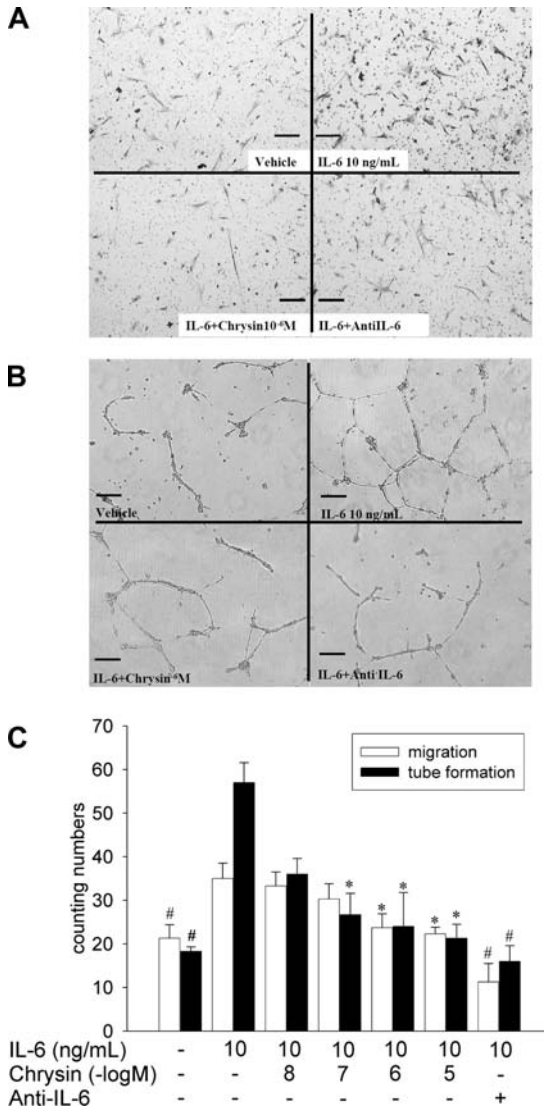

Figure 3. Effects of chrysin on phenotypic alterations of HUVECs. (**A**) Inhibitory effects of chrysin on endothelial cell migration. Cells were treated with chrysin (10^{-6} M) after IL-6 (10 ng/mL) induction for 18 h. (**B**) Inhibitory effects of chrysin on new vessel and network formation. Cells were treated with either chrysin (10^{-6} M) or neutralized IL-6 antibody (5 μg/mL, positive control) after IL-6 (10 ng/mL) induction for 18 h. The bar indicates 0.1 mm. (**C**) Quantization of the assays of endothelial cell migration and new vessel and network formation. $*$, $p < 0.05$ versus IL-6-treated group (one-way ANOVA); #, $p < 0.05$ versus IL-6-treated group (Wilcoxon rank sum test).

A previous study reported that chrysin exhibits weak inhibitory effects on angiogenesis, including tube formation and endothelial cell proliferation, in unstimulated cells (*19*). However, chrysin may show different effects on angiogenesis under various pathological conditions. Previously, we have demonstrated that chrysin strongly suppresses lipopolysaccharide (LPS)-induced angiogenesis in vitro and in vivo (*20*). Leung et al. have indicated LPS could activate retinal pigment epithelial cell and induce the expression of IL-6, IL-8, IL-6R, and IL-8RA (*21*). A previous study also reported that chrysin could inhibit insulin-induced VEGF expression in human prostate cancer cells (DU145) and could further inhibit DU145 xenograft induced-angiogenesis in mice (*22*). In this study, we verified that chrysin strongly suppressed VEGF expression when endothelial cells were exposed to IL-6. Using a CAM assay, we revealed that chrysin exhibited a powerful capacity of antiangiogenesis in cells

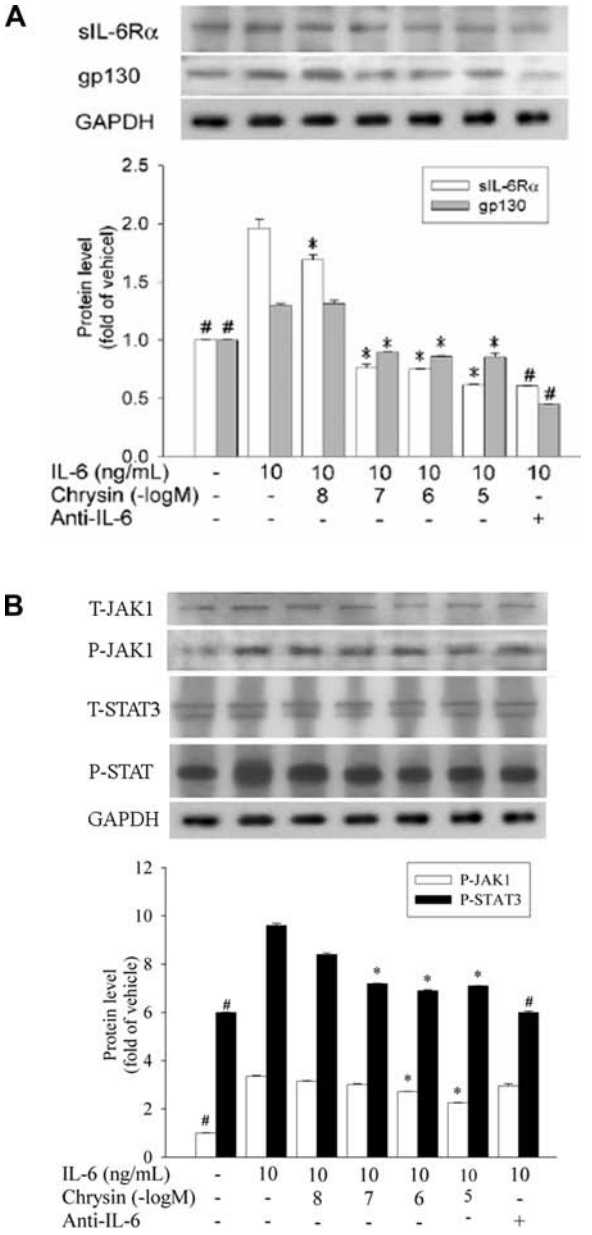

Figure 4. Inhibitory effects of chrysin on both soluble IL-6 receptor/gp130 and JAK1/STAT3 expressions in HUVECs. Chrysin attenuated soluble IL-6R/gp130 (**A**) and phosphorylated JAK1/STAT3 (**B**) protein expressions after IL-6 (10 ng/mL) induction for 12 h in a concentration-dependent manner. The assay was done in triplicate, and each sample was normalized to cells incubated without stimulation to determine the ratio of protein expression. ∗, $p < 0.05$ versus IL-6-treated group (one-way ANOVA); #, $p < 0.05$ versus IL-6-treated group (Wilcoxon rank sum test).

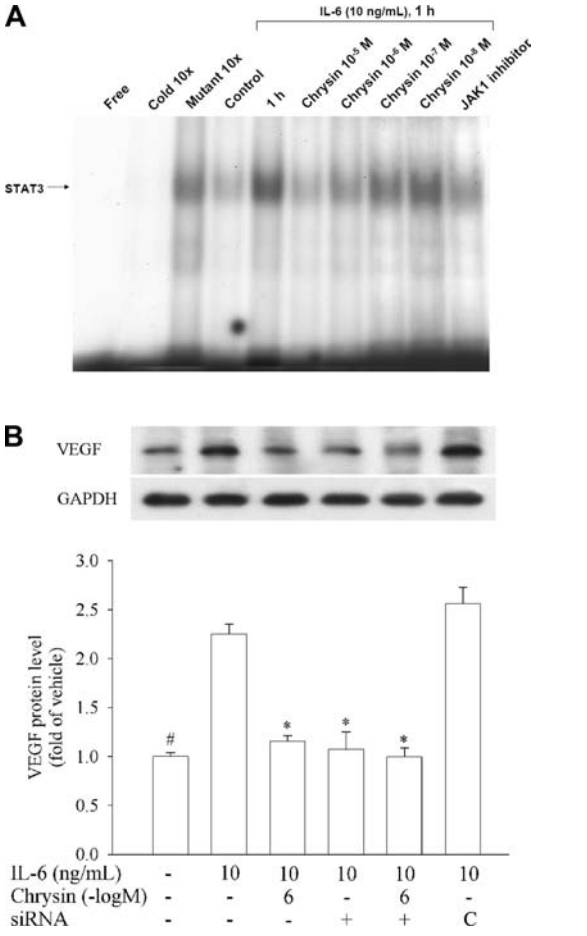

Figure 5. Role of STAT3 in chrysin-treated HUVECs. (**A**) EMSA analysis revealed that STAT3 binding activity was attenuated in a concentration-dependent manner by the addition of chrysin following a 1 h induction by IL-6 (10 ng/mL). (**B**) VEGF protein expression was attenuated by introducing siRNA for STAT3 either with IL-6 (10 ng/mL) or in combination with chrysin. In combination with siRNA, there was no further increase in the inhibition of VEGF protein in chrysin-treated HUVEC cultures. ∗, $p < 0.05$ versus IL-6 group; #, $p < 0.05$ versus IL-6 group (Wilcoxon rank sum test). (C: Control for siRNA).

induced by IL-6 in ovo, proving that this flavonoid blocked angiogenesis related to several pathological processes. Additionally, an MTT assay showed that chrysin did not reveal cytotoxicity in HUVECs and also had a suppressive effect on inflammatory lymphocytes with distinct structural requirements (*23*). We suspected that, in a normal microenvironment without an inducer, chrysin may not affect vascular development, but may block pathological angiogenesis induced by LPS, IL-6, or hypoxia. Therefore, chrysin may be a potential candidate for both preventive and therapeutic strategies. In contrast, Peng et al. reported two types of chrysin derivatives, coupled with NO donors of alkyl nitrate and furazan derivatives, promoted angiogenesis (*24*). Chrysin and its derivatives may provide new insight into the development of therapies to modulate angiogenesis.

The modulation of inflammation and oxidation of chrysin might play an important role responsible for this antiangiogenic effect of chrysin. Increasing amounts of evidence reveal that flavonoids, including chrysin, have many effects, including antiangiogenic, anti-inflammatory, and antioxidant activities, which are interconnected (*25*−*27*). Flavonoids were found to contain a phenolic hydroxyl (−OH) structure that is responsible for part of their antioxidant effects, which might contribute to their antiangiogenic activities, but the structural features of the phenolic hydroxy group among different flavonoids might affect their activities (*26*). Ahn et al. studied seven flavonoids in propolis to clarify the correlation between the antioxidant and antiangiogenic activities and found that chrysin tended to show relatively higher antiangiogenic activity than antioxidant activity (*19*). Furthermore, chrysin has also shown that it has anti-inflammatory effects in various kinds of cells, which leads to a potential clinical implication. Shin et al. showed that chrysin could improve murine inflammatory bowel disease through suppression of myeloperoxidase activity and several kinds of inflammatory mediators, including nitric oxide, prostaglandin E2, and

pro-inflammatory cytokines (*28*). In addition, Woo et al. also indicated that chrysin suppresses lipopolysaccharide-induced cyclooxygenase-2 expression through the inhibition of nuclear factor for IL-6 (NF-IL6) DNA-binding activity in macrophages (*29*). In our study, we demonstrated that chrysin regulates STAT3 and VEGF signaling in HUVECs incubated with IL-6. These results correspond to an antiangiogenic effect and inflammation regulation activity of chrysin.

STAT3 plays an important role in the sophisticated and coordinated processes of angiogenesis and inflammation, which are involved in tumor metastasis (*30*,*31*). Phosphorylated STAT3 is thought to be a useful indicator by which to study the pharmacodynamics of targeted angiogenesis inhibitors (*11*). In our study, chrysin down-regulated the amount and binding activity of phosphorylated STAT3 induced by IL-6. Furthermore, chrysin did not show more suppression on VEGF after STAT3 was knocked down, indicating a key role for STAT3 in this antiangiogenetic activity. Masuda et al. reported that epigallocatechin-3-gallate (EGCG), another flavonoid, also decreased VEGF production by inhibiting the constitutive activation of both STAT3 and NF-κB in head, neck, and breast carcinoma cells (*32*). Flavonoids such as EGCG have been reported to protect against oxidized LDL through hampering MAPK-dependent pathways involved in the activation of JAK-STAT in human endothelial cells (*33*). The flavonoid silibinin suppressed phosphorylated STAT3 and up-regulated the angiogenic inhibitors Ang-2 and Tie-2, leading to antiangiogenesis and lung cancer suppression in mice (*34*). In prostate cancer cells, radiation-induced STAT3 phosphorylation was suppressed by soy isoflavones, which led to antiangiogenesis (*35*). Taken together, we suggest that STAT3 plays a major role in the pharmacological mechanisms of flavonoids, especially in vascular development.

In conclusion, IL-6-induced endothelial activation and subsequent angiogenesis were abolished by chrysin both in vitro and in ovo. The antiangiogenic activity of chrysin was mediated by down-regulation of gp130/JAK1/STAT3/VEGF expression. Although the in ovo antiangiogenesis effects of chrysin were shown using a CAM assay, further studies in different in vivo models should be carried out to provide more information for clinical applications. Besides, because many of the studies had been done with incubation of pure molecules without taking into account metabolism, our results might further lead to other newly synthesized derivatives of chrysin with antiangiogenic effects. These results and previous studies suggest that chrysin may provide a therapeutic tool for the treatment of overangiogenic disorders.

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