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Ferulic acid augments angiogenesis via VEGF, PDGF and HIF-1α

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Abstract

Therapeutic angiogenesis is critical to wound healing and ischemic diseases such as myocardial infarction and stroke. For development of therapeutic agents, a search for new angiogenic agents is the key. Ferulic acid, a phytochemical found in many fruits and vegetables, exhibits a broad range of therapeutic effects on human diseases, including diabetes and cancer. This study investigated the augmenting effect of ferulic acid on angiogenesis through functional modulation of endothelial cells. Through endothelial cell migration and tube formation assays, ferulic acid (10^{-6}–10^{-4} M) was found to induce significant angiogenesis in human umbilical vein endothelial cells (HUVECs) *in vitro* without cytotoxicity. With chorioallantoic membrane assay, ferulic acid (10^{-6}–10^{-5} M) was also found to promote neovascularization *in vivo*. Using Western blot analysis and quantitative real-time polymerase chain reaction, we found that ferulic acid increased vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) expression in HUVECs. Furthermore, the amounts of hypoxic-induced factor (HIF) 1α mRNA and protein, the major regulator of VEGF and PDGF, also showed up-regulation by ferulic acid. Electrophoretic migration shift assay showed that the binding activity of HIF-1α was also enhanced with ferulic acid treatment of HUVECs. Moreover, inhibitors of extracellular-signal-regulated kinase 1/2 and phosphoinositide-3 kinase (PI3K) abolished the binding activity of HIF-1α and the subsequent activation of VEGF and PDGF production by ferulic acid. Thus, both mitogen-activated protein kinase and PI3K pathways were involved in the angiogenic effects of ferulic acid. Taken together, ferulic acid serves as an angiogenic agent to augment angiogenesis both *in vitro* and *in vivo*. This effect might be observed through the modulation of VEGF, PDGF and HIF-1α.
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Keywords: Ferulic acid; Angiogenesis; VEGF; PDGF; HIF-1α

1. Introduction

Angiogenesis, the formation of new blood vessels from preexisting capillaries to provide sufficient nutrients and oxygen, is an important process in embryonic development, wound healing and cardiovascular diseases [1]. Ischemic diseases, especially ischemic heart disease, remain a major and well-researched challenge for humans. In the process of angiogenesis, modulation of endothelial cells plays a key role in such processes as proliferation, migration and assembly. Numerous regulatory angiogenic factors have been identified, and their molecular modulations have been associated with several angiogenic disorders [2,3]. Although increasing evidence indicates that angiogenesis is a highly sophisticated and coordinated process, the activation of a hypoxic-induced factor (HIF) growth factor pathway remains the key modulator, especially in endothelial cells [4–6]. The survey and development of new agents promoting angiogenesis via these growth factors have become a focus of therapeutic strategies for these ischemic diseases. The impacts of

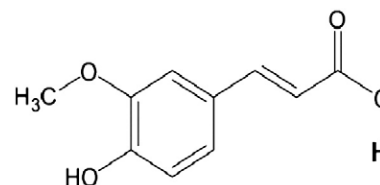

Fig. 1. Structure of ferulic acid.

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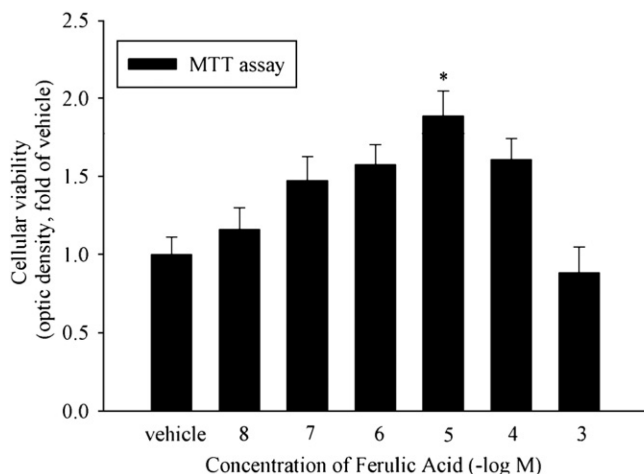

Fig. 2. Cytotoxicity of ferulic acid to HUVECs. The MTT assay was used to evaluate cytotoxicity. HUVECs (1500 cells/well in a 96-well plate) were treated with the indicated concentration of ferulic acid or vehicle for 48 h. Data are presented as the fold of vehicle group, which was set to 1. The assay was performed six times. *P<.05 versus vehicle group.

natural products on the cardiovascular promotion of angiogenesis or HIF autoregulated vascular endothelial growth factor (VEGF) gene expression have been increasingly studied, but the angiogenic effects of phytochemicals remain unclear [7,8].

Previous studies have shown that consumption of polyphenol compounds reveals an inverse relationship with the risk of cardiovascular disease, as well as chemopreventive effects [8–10]. Ferulic acid (Fig. 1) is an important polyphenol that is commonly found in vegetables and fruits such as sweet corn, rice bran and tomatoes, and it has been widely used as an immune-promoting agent in the treatment of blood deficiency diseases in Asia. Additionally, sodium ferulate has demonstrated antithrombotic and antioxidant activities in animal and human studies [11]. Thus, it is a potential agent for the treatment of neurodegenerative disorders, cardiovascular diseases, diabetes and several age-related disorders [9,12]. However, the potential effects of ferulic acid on angiogenic activity remain to be clarified. This study aims to investigate the molecular mechanisms and effects of ferulic acid on angiogenesis. We studied the ability of ferulic acid to modulate human umbilical vein endothelial cells (HUVECs) via cell migration and tube formation studies. Additionally, we examined the angiogenic effects of ferulic acid *in vivo* using chorioallantoic membrane (CAM) assay and investigated its effects on the expression and binding regulation of several important angiogenic factors [VEGF, platelet-derived growth factor (PDGF) and HIF-1α]. Finally, the potential roles of mitogen-activated protein kinase (MAPK) and phosphoinositide-3 kinase (PI3K) in the angiogenic effects of ferulic acid were also examined.

2. Materials and methods

2.1. Chemicals and reagents

High-purity (>98%) ferulic acid, dimethyl sulfoxide, penicillin, streptomycin and methylthiazole tetrazolium [MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Wortmannin and PD98059 were obtained from Calbiochem EMD Chemical, Inc. (Darmstadt, Germany). Basement membrane matrix (Matrigel) was acquired from Becton Dickinson (Bedford, MA, USA). Endothelial-cell-based medium (EBM) was purchased from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD, USA), and Medium 199 (M199) was purchased from GIBCO BRL (Gaithersburg, MD, USA). Antibodies for VEGF, PDGF and HIF-1α were purchased from R&D Systems (Minneapolis, MN, USA).

2.2. HUVEC cultures

HUVECs were isolated from human umbilical veins without underlying maternal diseases using a previously described method [13,14]. Cells were maintained in EBM and M199 containing 12% heat-inactivated fetal bovine serum (FBS), 100 μg/ml

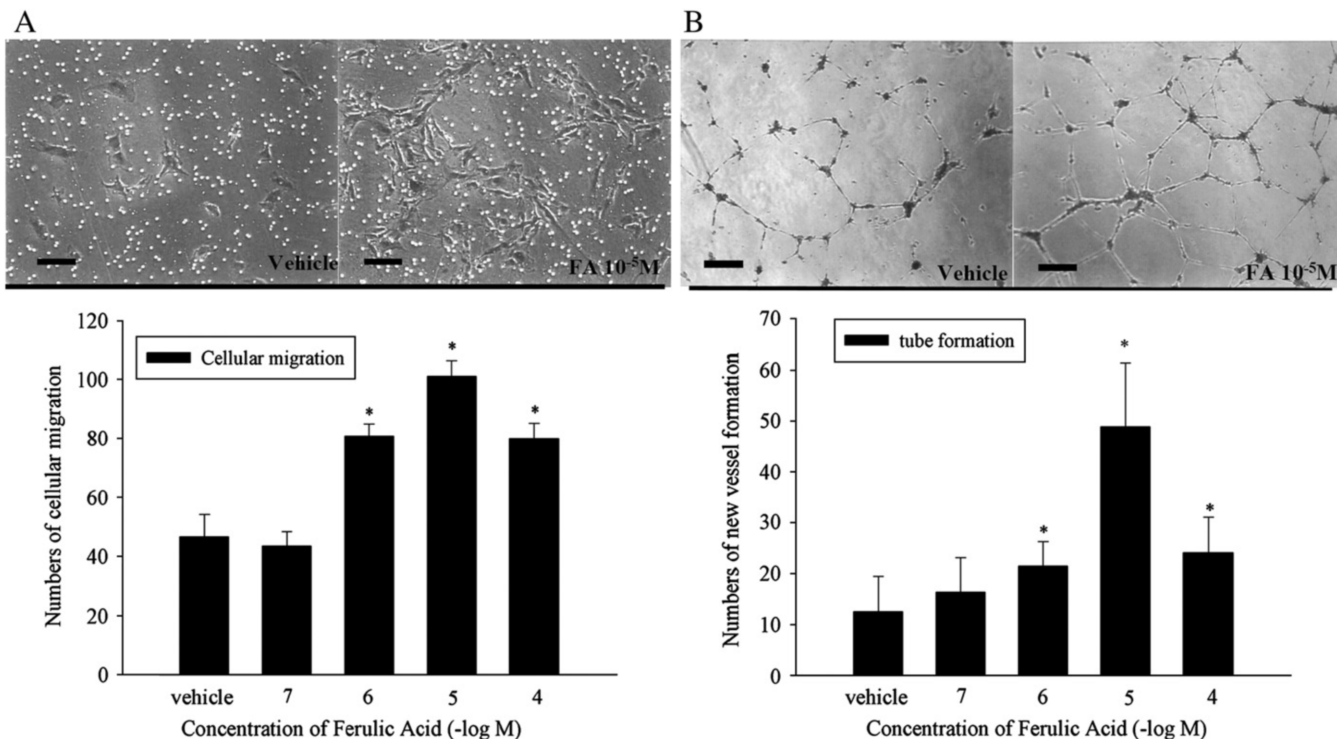

Fig. 3. Phenotypic alternation induced by ferulic acid in HUVEC cultures. Cell migration (A) and tube formation (B) were used as indicators of the phenotypic alteration of HUVECs. Cells (5×10^4) were treated with the indicated concentration of ferulic acid or vehicle for 18 h. The data were obtained from three independent experiments, and there were six repetitions with similar results. *P<.05 versus vehicle group. Scale bars indicate 0.1 mm.

penicillin G and 100 μg/ml streptomycin. In each independent experiment, HUVECs grown before the fifth passage were used for the studies. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Before each independent experiment, the amount of FBS in the media was decreased from 12% to 2%.

2.3. MTT assay for cell viability

To determine the potential cytotoxicity of ferulic acid on endothelial cells, HUVECs (1500 cells/well) were seeded overnight on a 96-well plate and allowed to incubate for 48 h with fresh EBM and M199 plus 2% FBS. Then, various concentrations of ferulic acid were added for 48 h at 37°C, followed by MTT assay to evaluate cell viability.

2.4. Cell migration assay

The HUVEC migration assay was performed as previously described, with minor modifications [14–16]. Briefly, 250 μl of Matrigel was coated onto each well of a 24-well multiple-plate Boyden chamber (BW 200s; Neuroprobe, Cain John, MD, USA) with polycarbonate filters (pore size, 8 μm) and then incubated at 37°C for 30 min. Then, 5×10^4 HUVECs were implanted onto each upper Boyden chamber's well, and ferulic-acid-treated medium was filled in the lower chamber to a total volume of 1 ml. After 18 h of incubation at 37°C and 5% CO_2, the Matrigel was removed. Then, cells that migrated to the reverse side of the filter (upper chamber) were stained with Liu's stain solution (Handsel Technologies, Inc., Taipei, Taiwan). Following a photograph of four randomized visual fields, the percentage of cell migration was measured by counting the number of endothelial cells that migrated through the membrane. The data are presented as percentage of control.

2.5. Tube formation assay

Tube formation assay was evaluated as previously described, with minor modifications [17]. By using the abovementioned Matrigel preparation in a Boyden chamber, HUVECs were cultured for 18 h at 37°C and 5% CO_2, followed by three-dimensional photographing with a revert-phase-contrast photomicroscope (Eclipase, TE2000-U; Nikon). Tube formation in HUVEC culture was quantified by counting tube-like structures in a gel, and data are presented as the number of branches per field (×100). In each experiment, four fields were calculated simultaneously, and four to six independent experiments were performed [18].

2.6. CAM in vivo

CAM assay was used for neovascularization tests *in vivo* [19]. Fertilized chicken embryos were incubated for 9 days at 38°C with 70% humidity for this assay. Two very small holes were drilled over the air sac at the end of the egg and into another zone of the egg, with suction performed to draft the air sac to the other hole. A small 1×1 cm window in the shell through the hole above the new air sac was made to expose the CAM. Straws (0.8 cm in diameter, 0.4 cm in height) were sterilized and put into the 1×1 cm window under laminar flow, and a 150-μl testing solution (vehicle and ferulic acid) was put into the straw on the CAM surface. Windows were sealed with transparent tape, and the eggs were incubated for 48 h. Blood vessels were counted and photographed with a Nikon digital camera, with two independent investigators counting the blood vessels for each tested group [20].

2.7. RNA isolation and real-time polymerase chain reaction (PCR)

All RNAs were isolated from HUVECs using Trizol reagent (Sigma-Aldrich Chemical Co.), in accordance with the manufacturer's directions. Lightcycler (Roche Diagnostics, Germany) was used for real-time PCR. The following primers were used for cDNA amplification: for VEGF, 5′-CATCGACAGAACAGTCCT-3′ (forward) and 5′-CAACTCAAGTCCACAGC-3′ (reverse; 386 bp); for PDGF, 5′-GTCCTAGAGCGTGTGG-3′ (forward) and 5′-CGCCGTGCCTACTAGA-3′ (reverse); for HIF-1α, 5′-AGTGTACCCTAACTAGCCG-3′ (forward) and 5′-ATCCTGTACTGTCCTGTGG-3′ (reverse; 405 bp); and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; internal control), 5′-CATCACCATCTTCCAGGAGC-3′ (forward) and 5′-GGATGATGTTCTGGGCTGCC-3′ (reverse; 405 bp). These were purchased from Mission Biotech Co. (Taipei, Taiwan).

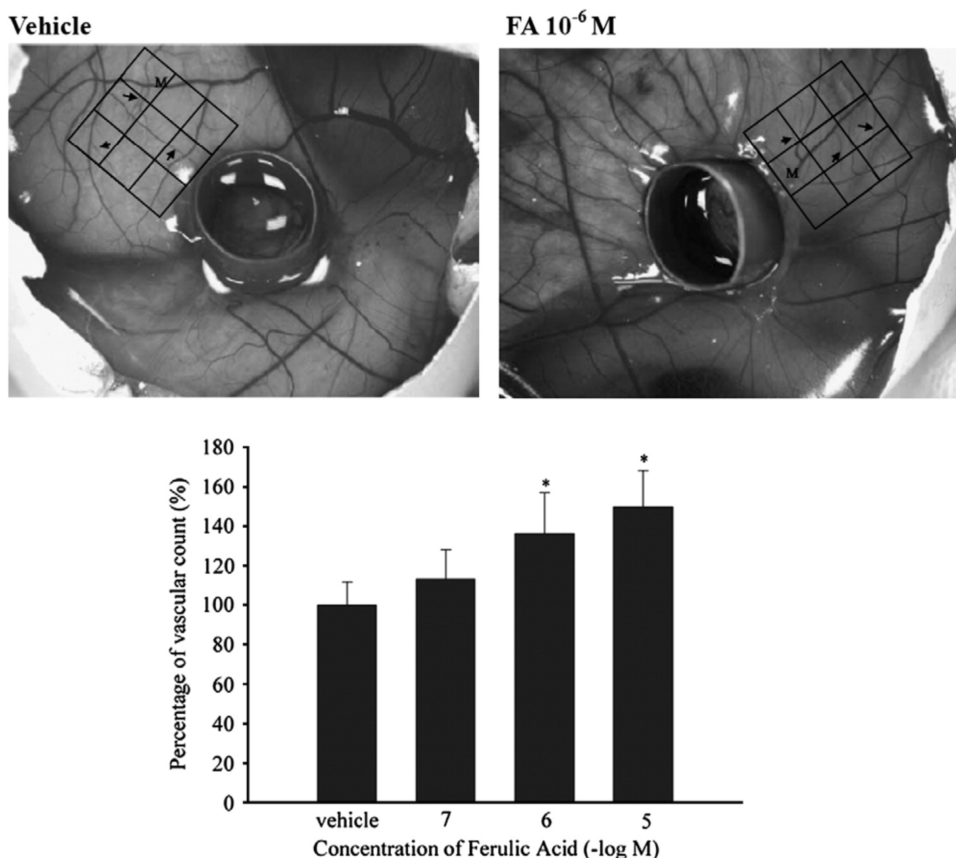

Fig. 4. Angiogenic effects of ferulic acid on chicken CAM. A representative photograph of CAM treated with vehicle and ferulic acid (10^{-6} M) is shown in the upper panel. The lower panel shows the quantification of neovascularization in the CAM model. The new vascular formation was calculated by crossing over the lines of a 3×3 chi-square area. All photographs were taken at 8× microscopic power field. Data are presented as the percentage of the vehicle group, which was set to 100%. Six eggs were included in each independent experiment. The assay was performed six times. *P<.05 versus vehicle group.

2.8. Western blot analysis for VEGF, PDGF and HIF-1α

Nuclear extracts were prepared from HUVECs as previously described [21]. Protein concentrations of nuclear extracts were measured with the Bio-Rad protein assay (Bio-Rad, USA). Nuclear proteins (30 μg) were separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose filters (Amersham Bioscience, Little Chalfont, UK) and probed with VEGF, PDGF or HIF-1α polyclonal antibodies. Signals were detected using enhanced chemiluminescence (Amersham Bioscience).

2.9. Electrophoretic migration shift assay

Electrophoretic mobility shift assays were performed as described previously [22] using the HIF-1α consensus (5′-CTGCATACGTGGGCTCCA-3′) and a mutant (5′-CTGCATAaaaGGGCTCCA-3′) as oligonucleotides. After these oligonucleotides had been annealed as double-stranded probes, they were labeled with [γ^{32}P]ATP using T4 polynucleotide kinase (Mission Biotech Co.). Control was performed with mutant oligonucleotides to compete with the labeled sequence. The binding reaction contains 20,000 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 $MgCl_2$ and 0.45 M NaCl+5 μg of nuclear proteins collected from different experimental conditions. Protein–DNA complex formation was carried out on ice for 20 min and resolved using 5% nondenaturing polyacrylamide gel electrophoresis, using 0.5 μg of Tris–borate–EDTA buffer (44.5 mM Tris base, 44.5 mM boric acid and 1 mM EDTA, pH 8.0) at 4°C. Gels were dried, and bands were detected using autoradiographic films.

2.10. Statistical analysis

All data in the different experimental groups are expressed as mean± S.D. and obtained from at least three independent experiments. Differences between groups were calculated by analysis of variance, followed by Duncan's multiple-range test or Student's *t* test, where appropriate. $P<.05$ was considered significant.

3. Results

3.1. Ferulic acid does not show cytotoxicity to endothelial cells

Using the MTT assay, treatment with ferulic acid (10^{-8}–10^{-3} M) for 48 h did not show cytotoxicity to endothelial cells. Furthermore, ferulic acid enhanced the proliferation of endothelial cells, reaching a maximum at 10^{-5} M (Fig. 2).

3.2. Effects of ferulic acid on the phenotypic modulation of endothelial cells

A cell migration assay and a tube formation assay were used to evaluate phenotypic alterations of endothelial cells as angiogenic parameters *in vitro*. By using VEGF (10 ng/ml) as positive control, ferulic acid (10^{-6}–10^{-4} M) induced significant endothelial cell migration (Fig. 3A) and tube formation (Fig. 3B) after 18 h of treatment.

3.3. Angiogenic effect of ferulic acid accessed via CAM assay

The CAM assay was designed to evaluate angiogenic effects *in vivo*. New vessel formation after treatment with ferulic acid (10^{-8}–10^{-5}M) was assessed, and ferulic acid (10^{-8}–10^{-5}M) was found to enhance new vessel formation in the CAM model (Fig. 4).

3.4. Ferulic acid induces the expression of VEGF, PDGF and HIF-1α in endothelial cells

We studied the effects of ferulic acid on VEGF and PDGF expression in HUVECs using concentration-dependent and time-course experiments. Treatment with a median concentration (10^{-5} M) of ferulic acid led to significant inductions of VEGF, PDGF and HIF-1α mRNA and protein expression, reaching maxima at 4 and 6 h, respectively (Figs. 5 and 6). Real-time quantitative PCR demonstrated definite increases in VEGF and PDGF mRNAs after 4 h of incubation (data not shown) after treatment with ferulic acid (10^{-8}–10^{-5} M). Thus, ferulic acid could induce the expression of major angiogenic regulatory factors, further promoting angiogenesis.

3.5. Ferulic acid augments HIF-1α binding activity in endothelial cells

To investigate the upstream mechanisms of VEGF and PDGF induction by ferulic acid, the binding activity of the major transcriptional factor HIF-1α was determined by electrophoretic

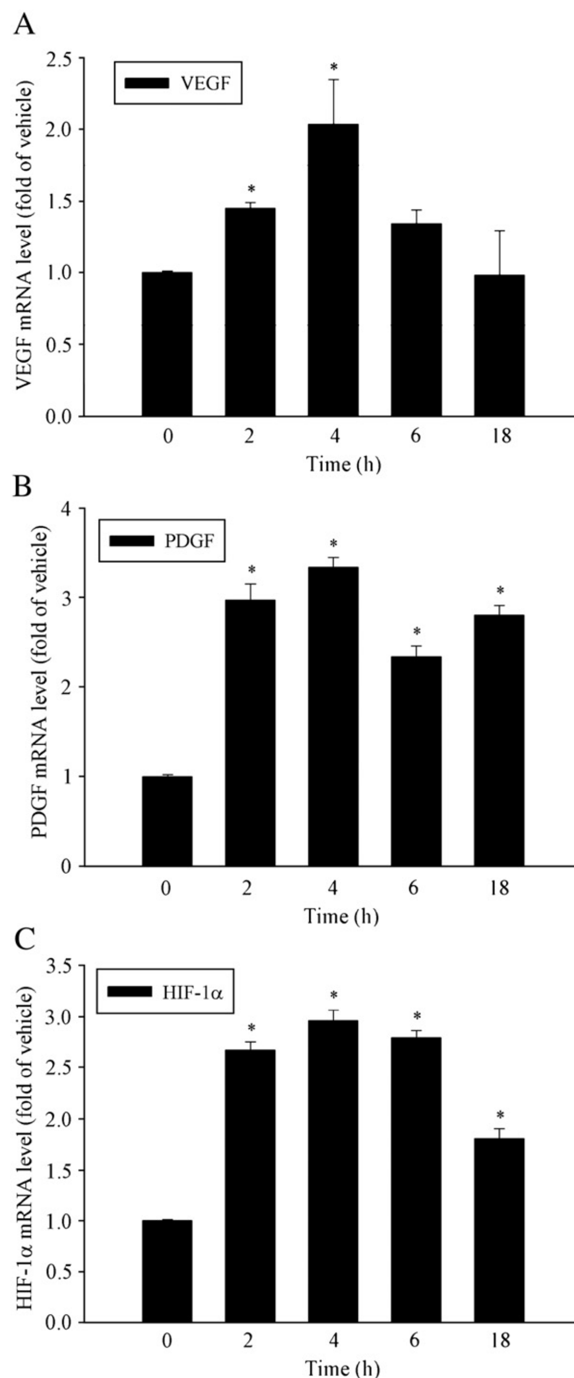

Fig. 5. Effects of ferulic acid on mRNA expressions of VEGF (A), PDGF (B) and HIF-1α (C). The mRNA levels of these three factors were quantified by real-time PCR, with gene expression normalized to GAPDH. HUVECs (5×10^4 cells) were treated with ferulic acid (10^{-5} M) for the indicated amount of time. Data are presented as the fold of the vehicle group, with the mRNA level of the group without treatment set to 1. The assay was performed in triplicate and repeated six times. *$P<.05$ versus vehicle group.

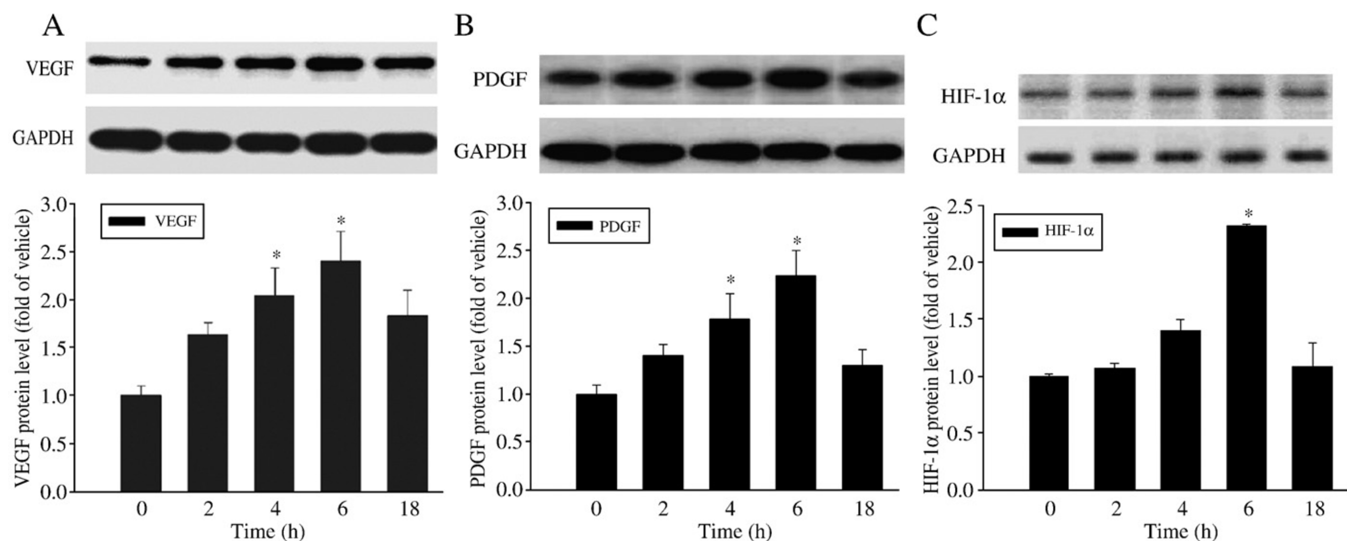

Fig. 6. Effects of ferulic acid on the protein expressions of VEGF (A), PDGF (B) and HIF-1α (C). Western blot analysis was used to analyze the protein expression of these factors in whole-cell extracts of HUVECs normalized to GAPDH. Cells (5×10^4) were treated with ferulic acid (10^{-5} M) for the indicated amount of time. Data are presented as the fold difference of the group without treatment, which was set to 1. The assay was performed in triplicate and repeated six times. *P<.05 versus group without treatment.

migration shift assay. In this assay, HIF-1α binding activity in HUVECs was induced by ferulic acid (10^{-5} M), reaching a peak at 2 h (Fig. 7). These results suggest that ferulic acid enhances the binding of HIF-1α, in addition to up-regulating its expression and subsequently increasing the expression of downstream genes such as *VEGF* and *PDGF*.

3.6. Angiogenic effects of ferulic acid are observed through the MAPK and PI3K pathways

To study the roles of the MAPK and PI3K pathways in the angiogenic effects of ferulic acid, HUVECs were pretreated with PD98059 (a MAPK inhibitor) or Wortmannin (a PI3K inhibitor). After PD98059 or Wortmannin treatment, the binding activity of HIF-1α induced by ferulic acid decreased (Fig. 7), and the expressions of ferulic-acid-induced VEGF and PDGF were also repressed (Fig. 8). These results suggest that ferulic-acid-mediated angiogenesis occurs through the MAPK and PI3K pathways.

4. Discussion

In this study, we demonstrated for the first time that ferulic acid exhibits angiogenic activity both *in vitro* and *in vivo* via stimulation of the VEGF, PDGF and HIF-1α pathways. We also showed that the angiogenic effects of ferulic acid occurred through the MAPK and PI3K pathways. Ferulic acid plays a novel role in angiogenic effects and is a potential new therapeutic agent for ischemic diseases.

Polyphenols are a family of natural compounds that are widely distributed in plant foods. Ferulic acid is a polyphenol derived from some fruits and Chinese herbs such as red grapes and *Angelica sinensis* [23]. There is evidence suggesting that polyphenols, including ferulic acid, have tumor-suppression potential in breast cancer cell lines and also have antioxidant activities [24,25]. However, among polyphenols, our results showed that the role of ferulic acid in angiogenesis is similar to that of salvianolic acid B [26]. In contrast, several common polyphenols act as angiogenesis inhibitors, such as epigallocatechin-3-gallate in green tea and resveratrol in red wine [27].

With regard to the modulation of endothelial cells, which is a key step of angiogenesis, our results revealed that ferulic acid has significant stimulatory effects on cellular migration and tube formation, a typical response to angiogenic stimulation. Furthermore, our findings of enhancement by ferulic acid of the expression levels of several angiogenesis-related factors, including VEGF, PDGF and HIF-1α, show that ferulic acid enriches the angiogenic effect on HUVECs. However, Hou et al. [28] have previously shown the opposite result — that ferulic acid attenuated endothelial cell proliferation and cell cycle progression through nitric oxide down-regulation of the extracellular-signal-regulated kinase (ERK) 1/2 pathway in the ECV304 cell line. However, the ECV304 cell line, which is not of HUVEC origin, has been recently recognized as a possible bladder cancer cell line and not as an appropriate cell line for the study of endothelial cell biology [29].

Recent investigations concerning the anti-cardiovascular disease properties of many polyphenolic compounds, such as quercetin and resveratrol, have demonstrated up-regulation of endothelial nitric oxide synthase (eNOS) gene expression in HUVEC cultures [8]. In

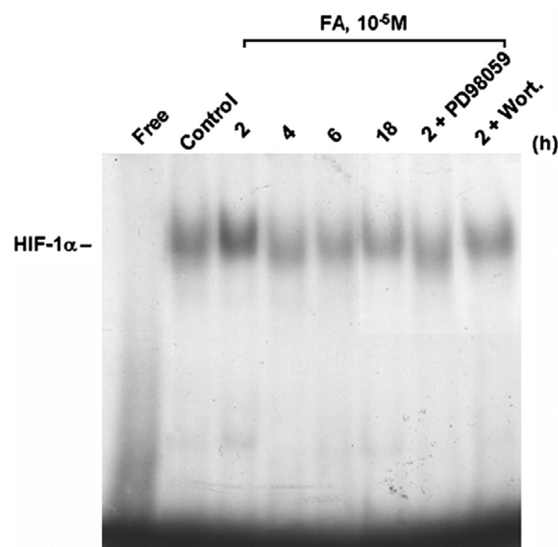

Fig. 7. Effects of ferulic acid on the binding activity of HIF-1α. HUVECs were treated without or with PD98059 (50 μM) or Wortmannin (50 nM) for 30 min, followed by ferulic acid (10^{-5} M) for the indicated time. Nuclear extracts (NE) harvested from HUVECs were incubated with ^{32}P-labeled HIF-1α consensus oligonucleotide as probe.

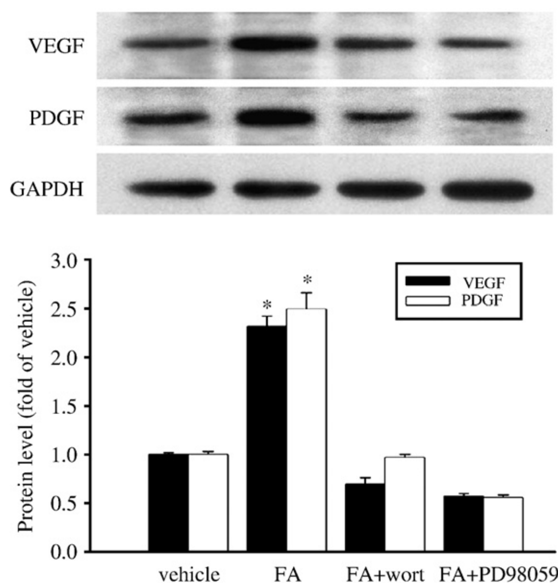

Fig. 8. Effects of the ERK inhibitor PD98059 and the PI3K inhibitor Wortmannin on ferulic-acid-induced VEGF and PDGF expression. The protein expression of VEGF and PDGF was determined by Western blot analysis. HUVECs were pretreated with or without PD98059 (50 μM) or Wortmannin (50 nM) for 30 min, followed by ferulic acid (10^{-5} M) for 6 h. Total cell extracts were analyzed by an immunoblot assay using VEGF or PDGF antibody. Densitometry was normalized to GAPDH. The assay was repeated six times. *P<.05 versus vehicle group.

comparison to the results of Nicholson et al., our results indicate that treatment with a physiological concentration (10^{-5} M) of ferulic acid revealed no significant effect on eNOS (data not shown). Our results did show significant changes in VEGF and PDGF protein expression levels in ferulic-acid-treated HUVECs. Based on our results, the transcriptional factor HIF-1α was the important upstream factor regulating VEGF and PDGF and had the same role in ferulic-acid-treated HUVEC cultures. Based on analyses of protein levels and mRNA expression, our data revealed that ferulic acid enhanced the *de novo* gene expression of VEGF and PDGF.

VEGF, PDGF and hypoxia enhance angiogenesis in response to neovascularization and correlate with ischemic diseases [30]. Zhang et al. [31] documented that four hydroxycinnamic acids (caffeic, ferulic, sinapic and chlorogenic acids) tested were less effective than three flavonoids (quercetin, rutin and catechin); furthermore, of all the compounds tested, quercetin offered the strongest protection against H_2O_2-induced cell death. Nevertheless, in this present study, ferulic acid augmented angiogenesis through the up-regulation of VEGF and PDGF pathways in a novel way. Our investigations of VEGF and HIF modulation indicate that the angiogenic effect occurs through MAPK, PI3K and HIF signal transcription. Taken together, these data provide evidence of a close relationship between HIF signaling and neovascularization, which is further supported by our results from the *in vivo* CAM model showing that new vessel formation was significantly augmented by ferulic acid treatment.

However, in addition to angiogenic effects, Yogeeta et al. [32] showed the synergistic potential of ferulic acid and ascorbic acid in mitochondrial dysfunctions during β-adrenergic catecholamine-induced cardiotoxicity and associated oxidative stress in rats. Interestingly, ferulic acid has diverse effects on the cardiovascular system under different conditions, suggesting caution when considering clinical applications.

In summary, ferulic acid enhances both *in vitro* and *in vivo* angiogeneses through modulation of the VEGF, PDGF and HIF-1α pathway. It may shed light on the clinical application of ferulic acid in ischemia-related diseases.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jnutbio.2009.04.001.

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