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Antiangiogenic properties of cafestol, a coffee diterpene, in human umbilical vein endothelial cells

Shuaiyu Wang [a,b], Yeo Cho Yoon [b], Mi-Jeong Sung [b], Haeng-Jeon Hur [b], Jae-Ho Park [b,*]

[a] Food Biotechnology, University of Science & Technology, 113 Gwahangno, Yuseong-gu, Daejeon 305-333, Republic of Korea
[b] Korea Food Research Institute, 516 Baekhyun-dong, Bundang-gu, Songnam, Kyungki-do 463-746, Republic of Korea

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ABSTRACT

As angiogenesis plays important roles in tumor growth and metastasis, searching for antiangiogenic compounds is a promising tactic for treating cancers. Cafestol, a diterpene found mainly in unfiltered coffee, provides benefit through varied biological activity, including antitumorigenic, antioxidative, and anti-inflammatory effects. This study aimed to investigate the effects of cafestol on angiogenesis and to uncover the associated mechanism. We show that cafestol inhibits angiogenesis of human umbilical vascular endothelial cells. This inhibition affects the following specific steps of the angiogenic process: proliferation, migration, and tube formation. The inhibitory effects of cafestol are accompanied by decreasing phosphorylation of FAK and Akt and by a decrease in nitric oxide production. Overall, cafestol inhibits angiogenesis by affecting the angiogenic signaling pathway.

1. Introduction

Angiogenesis takes place during development of embryo, tissue growth, and regeneration after injury to supply oxygen and nutrients [1]. Because blood vessels nourish all organs of the body, pathological angiogenesis is a crucial process in cancer and in various ischemic and inflammatory diseases [2]. It has been suggested that inhibiting or delaying angiogenesis is a promising strategy to treat cancer and several other diseases [3].

The formation of a mature vasculature is a highly regulated process that mainly involves endothelial cell proliferation, migration, tube formation, and recruitment of supporting cells. It requires a coordinated signaling pathway involving multiple extracellular factors and their respective cell surface receptors, as well as intracellular effectors [4].

On the extracellular level, vascular endothelial growth factor (VEGF) is the most important growth factor during angiogenesis. In endothelial cells, biologically relevant VEGF signaling is primarily mediated by VEGF receptor VEGFR2 [5]. Another significant player is focal adhesion kinase (FAK), a membrane-proximal signaling molecule. FAK is involved in the proliferation, migration, and survival of endothelial cells and has been shown to control angiogenesis in cancer [6]. Therefore, their downstream intermediates, such as Akt, Erk, and nitric oxide (NO), play important roles in angiogenesis [4]. An antiangiogenic strategy can target one or more of the various signaling components [7].

Coffee, a worldwide beverage, is a complex mixture of several compounds [8]. One of these is cafestol, a bioactive diterpene present mainly in unfiltered coffee beverages. It has been reported that cafestol exerts anticarcinogenic and antioxidant effects [9–11]. However, the effect of cafestol on angiogenesis has remained unclear. Here, we report that cafestol inhibits angiogenesis in human umbilical vascular endothelial cells (HUVECs) by blocking proliferation, migration, and tube formation. The inhibitory effects of cafestol are mediated through phosphorylation of FAK and Akt, along with decreased NO production.

2. Materials and methods

2.1. Materials

Cafestol was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). The Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA). The extracellular matrix (ECM) gel was purchased from Sigma (St. Louis, MO, USA). The antibodies directed against phospho-Erk (Thr202/Tyr204), phospho-VEGFR2, and phospho-Akt (Ser473) were purchased from Cell Signaling Technology (Danvers, MA, USA). The antibody directed against GAPDH was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the antibody directed against phospho-FAK was obtained from BD Biosciences (Bedford, MA, USA). A NO detection kit was purchased from iNtRON Biotechnology (Kyunggi, Korea). All the other reagents were purchased from Sigma, unless otherwise indicated.

* Corresponding author. Fax: +82 31 709 9876.
E-mail address: jaehoparkmail@gmail.com (J.-H. Park).

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2.2. Cell culture and proliferation assay

The HUVECs were purchased from the American Type Culture Collection (Manassas, VA, USA) and were maintained in DMEM supplemented with 10% FBS and 1% antibiotics at 37 °C and in an atmosphere containing 5% CO_2. The cells were split 1:3 when they reached 80–90% confluence. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. The cells (1×10^4 cells/well) with 10% FBS culture medium were seeded in a 24-well plate and incubated overnight. Next, the cells were starved for 6 h with 2% FBS-containing medium. Then, the cells were treated with various amounts of cafestol dissolved in 500 μL of 2% FBS medium and incubated for 24 h. Subsequently, 50 μL of 5-mg/mL MTT was transferred into each well, and the cells were incubated for 4 h. The medium in each well was carefully removed, and 500 μL DMSO was then added to each well. The samples were thoroughly agitated for 10 min on a shaker. Finally, the absorbance of the samples at 490 and 690 nm was measured against a background control (blank) using a microplate reader.

2.3. Transwell cell migration assay

As described previously [12], fresh DMEM containing 2% FBS and cafestol was placed in the lower wells. Cell suspensions were loaded into each of the upper wells (200 μL/well). The chamber was incubated at 37 °C for 6 h. After incubation, images of the cells that migrated into the lower well were captured with a phase-contrast microscope (Olympus, Tokyo, Japan). Cell migration was quantified by counting the number of cells in the lower well.

2.4. Wound healing assay

The HUVECs (1×10^5 cells/well) were cultured in 12-well plates rinsed with PBS and then starved overnight in 2% FBS medium until they reached 90% confluence. A single wound was then scratched in the center of the cell monolayers with a 200-μL sterile plastic pipette tip. The wounded monolayers were washed twice to remove the nonadherent cells and were incubated with various concentrations of cafestol for 21 h in the presence of 2 μg/mL of mitomycin C. To measure the length of the endothelial cells that had migrated from the edge of the injured monolayer, images were obtained immediately after wounding and after a 21-h incubation period, using a phase-contrast microscope (Olympus). The length was measured by the Image-Pro Plus v 6.0 (Media Cybernetics, Inc., Bethesda, MD, USA). Each experiment was repeated at least three times.

2.5. Capillary tube formation assay

ECM gel was added to a 96-well plate (50 μL/well) and was allowed to polymerize for 1 h at 37 °C. The HUVECs (3×10^4 cells/well) with and without cafestol, were seeded onto the surface of the ECM gel in the 96-well plate. After a 20-h incubation period, cellular morphological changes and tubular structure formation were observed under a phase-contrast microscope (Olympus). The images were captured and the degree of tube formation was quantified by measuring the lengths of the tubes in eight randomly chosen low-power (100×) fields using the Image-Pro Plus v 6.0.

2.6. Western blot analysis

The HUVECs (2×10^5 cells/well) were cultured in 6-well plates. When they reached 90% confluence, the cells were serum starved for 12 h in a medium containing 2% FBS and were then treated with cafestol for 30 min or 1.5 h. The cells were washed with ice-cold PBS and then lysed with lysis buffer (50-mM Tris–HCl, 1% Triton X-100, 0.5% sodium deoxycholate, 150-mM NaCl, 1-mM EDTA, 1-mM phenylmethylsulfonyl fluoride [PMSF], 1-mM sodium orthovanadate, 1-mM NaF, and 0.2% protease inhibitor cocktail; pH 7.2). Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and were subsequently transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk in 1× Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature and were then incubated overnight at 4 °C with a primary antibody. The following day, the membranes were washed with TBST and were probed with a secondary antibody. The bands were detected using enhanced chemiluminescence reagents (Amersham, Piscataway, NJ, USA). The signal intensities were determined by Quantity One 4.6.2 (Bio-Rad, Hercules, CA, USA) and were subjected to statistical analysis.

2.7. NO measurement

The NO levels in the HUVECs were measured with the NO detection kit. Briefly, the HUVECs were cultured in 6-well plates. After overnight incubation, the cells were treated with cafestol. After 6 h, the supernatant was collected, and NO production was determined following the protocol supplied with the kit.

2.8. Statistical analysis

All the experiments were performed in triplicate, and the data are presented as mean ± SD values. Differences between the mean values were assessed using 1-way analysis of variance. For all the analyses, $p < 0.05$ was considered significant. Statistical analyses were performed using SPSS 15.0 (SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Cafestol inhibits HUVEC proliferation

As endothelial cell proliferation is an essential event at the beginning of angiogenesis, we performed *in vitro* growth studies to assess the effect of cafestol on HUVEC proliferation. The HUVECs were treated with various concentrations of cafestol; then, the cell viability was measured by MTT assay. As shown in Fig. 1, up to 10-μM treatment with cafestol for 24 h did not inhibit HUVEC proliferation, whereas 20- to 80-μM cafestol inhibited proliferation in a dose-dependent manner.

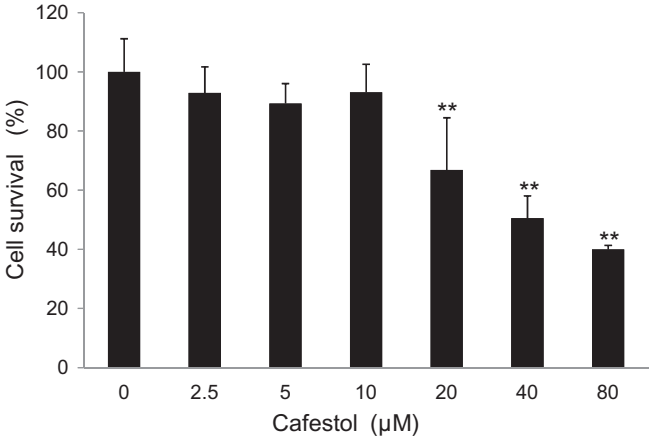

Fig. 1. Cafestol inhibits HUVEC proliferation. The HUVECs were treated with a series of cafestol concentrations, and after 24 h, cell proliferation was evaluated by MTT assay. The data shown in the graphs are the mean ± SD values from at least 3 individual experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ versus the controls.

3.2. Cafestol inhibits HUVEC migration

As migration of endothelial cells plays a central role in angiogenesis during tumor growth, we investigated the effects of cafestol on HUVEC migration. Using transwell plates, we found that HUVEC migration was significantly inhibited by 6-h treatment with 10- or 20-μM cafestol (Figs. 2A, Supplementation 1). To confirm the inhibition of HUVEC migration by cafestol, a wound healing assay was performed in the presence of 2-μg/mL mytomycin C. Consistent with the initial results (Fig. 2A, Supplementation 1), HUVEC migration was significantly inhibited by the 21-h treatment with 10- or 20-μM cafestol (Fig. 2B).

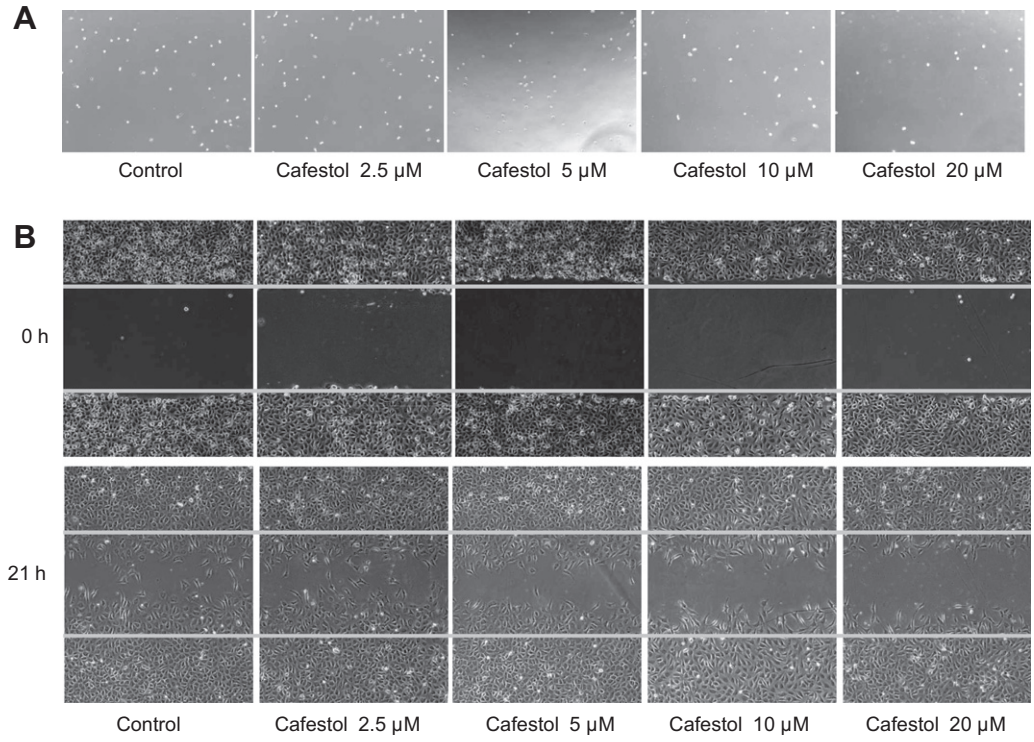

Fig. 2. Cafestol inhibits HUVEC migration. (A) Transwell migration assay: after incubation for 6 h, images were captured with a phase-contrast microscope. (B) Wound healing assay: after treatment with cafestol in the presence of 2-μg mitomycin C; HUVEC migration was recorded by microscopy at 24 h. Images of the wounded monolayers of the HUVECs.

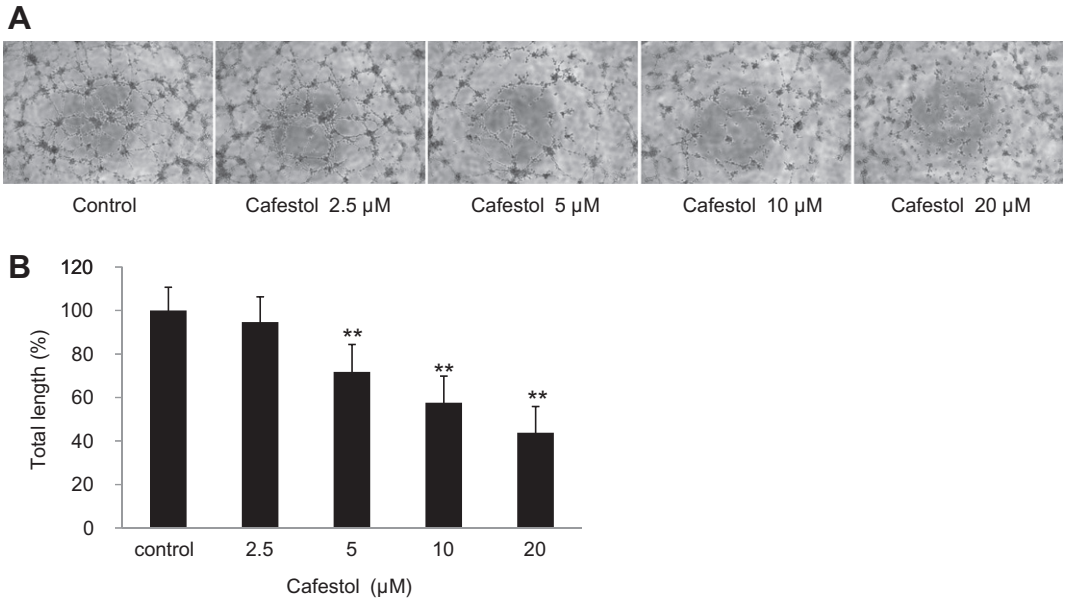

Fig. 3. Cafestol inhibits HUVEC tube formation. (A) HUVECs were seeded onto the Matrigel-coated 96-well plate with cafestol. After 20 h, the tube-like networks were photographed (100×). (B) The length of the tube networks was quantified using the Image-Pro Plus v 6.0 software. The data shown in the graphs are the mean ± SD values of at least three individual experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ versus control.

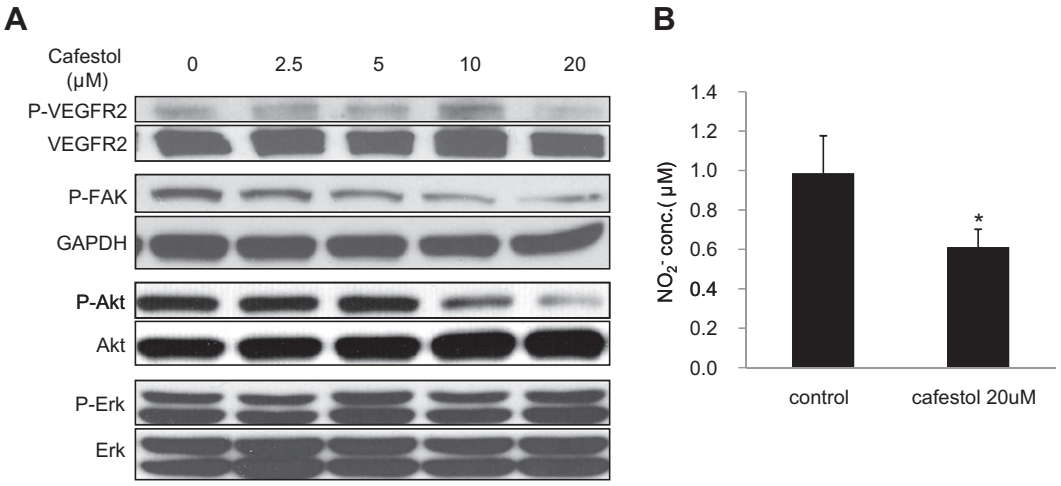

Fig. 4. Cafestol inhibits the phosphorylation of VEGFR-2, FAK, Akt, but does not affect Erk. HUVECs were incubated with 0–20 μM cafestol. The levels of phosphorylated and total proteins or GAPDH were determined by Western blot analysis. (A) Cafestol partially inhibits VEGFR-2 phosphorylation at 20 μM. Cafestol inhibits the phosphorylation of FAK, Akt in a dose-dependent manner. Phosphorylation of Erk is not affected by Cafestol. (B) Cafestol decrease NO production in HUVECs. HUVECs were incubated with 20 μM cafestol for 6 h. Nitrite concentration in the culture medium was determined using the nitric oxide (NO) detection kit. Data shown in the graph is the mean ± SD values of at least three individual experiments. $^*p < 0.05$ versus control.

3.3. Cafestol inhibits HUVEC tube formation on the Matrigel

An *in vitro* endothelial tube formation assay was performed in the absence or presence of cafestol. The total length of the endothelial tubes formed on the Matrigel was significantly reduced by cafestol in a dose-dependent manner (Fig. 3A and B).

3.4. Cafestol partially inhibits the phosphorylation of VEGFR2

As VEGF and its receptor VEGFR2 play essential roles in the angiogenesis signaling pathway, the effects of cafestol on VEGFR2 activation were carefully examined. As shown in Figs. 4A and Supplementation 2, the 20-μM cafestol reduced VEGFR2 phosphorylation, but lower cafestol concentrations did not. These results indicate that the inhibition of angiogenesis by cafestol is not mediated principally through VEGFR2 phosphorylation, suggesting that cafestol may also affect other antiangiogenic modulators in endothelial cells.

3.5. Cafestol inhibits the phosphorylation of FAK and Akt but does not affect the phosphorylation of the Erk1/2 MAPKs

FAK is a ubiquitously expressed nonreceptor protein tyrosine kinase. It has been reported that FAK is involved in endothelial cell migration, proliferation, and survival [6,13]. Figs. 4A and Supplementation 3 shows that treatment with cafestol for 1.5 h inhibits the phosphorylation of FAK in a dose-dependent manner.

Activation of Akt and the ERK1/2 MAPKs is thought to be essential for endothelial cell survival and proliferation [14,15]. To assess the effect of cafestol on these biological responses, a western blot assay was performed. Cafestol treatment significantly inhibited the phosphorylation of Akt in a dose-dependent manner. In contrast, up to 20-μM cafestol was unable to inhibit the phosphorylation of the ERK 1/2 MAPKs (Fig. 4A, Supplementation 4).

3.6. Cafestol inhibits endothelial cell nitric oxide production

NO potentiates angiogenesis by regulating endothelial cell survival and migration [16–20]. We assayed the NO production in the HUVECs. After treating the HUVECs with 20-μM cafestol for 6 h, the concentration of NO was significantly reduced (Fig. 4B).

4. Discussion

Because angiogenesis is a crucial process during tumor growth, developing antiangiogenic drugs is a strategy for cancer treatment [7]. However, the first generation of antiangiogenic molecules performed poorly in clinical applications [21]. Because tumor angiogenesis is a complex process and involves a number of cell types, a multitarget approach will likely show better results in clinical trials [22]. Here, we found that cafestol functions as an antiangiogenic agent by affecting several molecular processes, including phosphorylation of FAK and Akt, as well as NO production, in the HUVECs stimulated by VEGF.

Among the several regulatory and signaling molecules governing angiogenesis, VEGF and its receptor, VEGFR, play prominent roles. The VEGF-A isoform is a proangiogenic factor involved in cell migration, proliferation, and survival. VEGFR2 is known to mediate most of the angiogenic effects of VEGF-A [5]. We found that VEGFR2 phosphorylation was reduced by the 20-μM cafestol treatment but not by lower concentrations (Fig. 4A). However, the migration and tube formation of the HUVECs were significantly inhibited by 5- to 10-μM cafestol. In addition, the phosphorylations of FAK and Akt were significantly reduced in a dose-dependent manner. These results suggest that in the VEGF-stimulated HUVECs, cafestol has little effect on early angiogenesis signaling but mainly influences signaling pathways downstream of VEGF, resulting in inhibition of angiogenesis.

FAK is a membrane-proximal signaling molecule that mediates signals from integrins and from receptor tyrosine kinases to promote cell survival and migration [6]. A reduction of FAK levels leads to decreased VEGF expression, suppressing tumor angiogenesis [23]. We have demonstrated for the first time that cafestol inhibits the phosphorylation of FAK in VEGF-stimulated HUVECs. Together with the previous data showing inhibition of migration and tube formation by cafestol, our study suggests that the antiangiogenic activity of cafestol is linked to FAK inhibition.

Akt plays important roles in various aspects of angiogenesis, including cell survival, migration, tube formation, and NO

production [24]. In our study, cafestol reduced the phosphorylation of Akt and consequently inhibited the migration, tube formation, and NO production of the VEGF-stimulated HUVECs. Together, these findings suggest that cafestol affects the angiogenesis of HUVECs by regulating the phosphorylation of not only FAK but also Akt.

In summary, this study demonstrates that cafestol is a potent antiangiogenic phytochemical that exerts effects on several important molecular processes during angiogenesis, including the phosphorylation of FAK and Akt and the production of NO. Our findings indicate that cafestol may be exploited as a therapeutic antiangiogenic drug.

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.bbrc.2012.04.046.

References

[1] P. Carmeliet, R.K. Jain, Molecular mechanisms and clinical applications of angiogenesis, Nature 473 (7347) (2011) 298–307.
[2] P. Carmeliet, R.K. Jain, Angiogenesis in cancer and other diseases, Nature 407 (6801) (2000) 249–257.
[3] N. Ferrara, R.S. Kerbel, Angiogenesis as a therapeutic target, Nature 438 (7070) (2005) 967–974.
[4] S.M. Weis, D.A. Cheresh, Tumor angiogenesis: molecular pathways and therapeutic targets, Nat. Med. 17 (11) (2011) 1359–1370.
[5] N. Ferrara, H.P. Gerber, J. LeCouter, The biology of VEGF and its receptors, Nat. Med. 9 (6) (2003) 669–676.
[6] T. Lechertier, K. Hodivala-Dilke, Focal adhesion kinase and tumour angiogenesis, J. Pathol. 226 (2) (2012) 404–412.
[7] K.M. Cook, W.D. Figg, Angiogenesis inhibitors: current strategies and future prospects, CA Cancer J. Clin. 60 (4) (2010) 222–243.
[8] S.E. George, K. Ramalakshmi, L.J. Mohan, A perception on health benefits of coffee, Crit. Rev. Food. Sci. Nutr. 48 (5) (2008) 464–486.
[9] C. Cavin, D. Holzhaeuser, G. Scharf, et al., Cafestol and kahweol, two coffee specific diterpenes with anticarcinogenic activity, Food Chem. Toxicol. 40 (8) (2002) 1155–1163.
[10] K.J. Lee, J.H. Choi, H.G. Jeong, Hepatoprotective and antioxidant effects of the coffee diterpenes kahweol and cafestol on carbon tetrachloride-induced liver damage in mice, Food Chem. Toxicol. 45 (11) (2007) 2118–2125.
[11] K.J. Lee, H.G. Jeong, Protective effects of kahweol and cafestol against hydrogen peroxide-induced oxidative stress and DNA damage, Toxicol. Lett. 173 (2) (2007) 80–87.
[12] S.J. Lee, S. Namkoong, Y.M. Kim, et al., Fractalkine stimulates angiogenesis by activating the Raf-1/MEK/ERK- and PI3K/Akt/eNOS-dependent signal pathways, Am. J. Physiol. Heart Circ. Physiol. 291 (6) (2006) H2836–H2846.
[13] S.K. Hanks, T.R. Polte, Signaling through focal adhesion kinase, Bioessays 19 (2) (1997) 137–145.
[14] H.P. Gerber, A. McMurtrey, J. Kowalski, et al., Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation, J. Biol. Chem. 273 (46) (1998) 30336–30343.
[15] A. Parenti, L. Morbidelli, X.L. Cui, et al., Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase1/2 activation in postcapillary endothelium, J. Biol. Chem. 273 (7) (1998) 4220–4226.
[16] E. Noiri, Y. Hu, W.F. Bahou, et al., Permissive role of nitric oxide in endothelin-induced migration of endothelial cells, J. Biol. Chem. 272 (3) (1997) 1747–1752.
[17] M.S. Goligorsky, H. Abedi, E. Noiri, et al., Nitric oxide modulation of focal adhesions in endothelial cells, Am. J. Physiol. 276 (6 Pt 1) (1999) C1271–C1281.
[18] S. Dimmeler, E. Dernbach, A.M. Zeiher, Phosphorylation of the endothelial nitric oxide synthase at ser-1177 is required for VEGF-induced endothelial cell migration, FEBS Lett. 477 (3) (2000) 258–262.
[19] S. Dimmeler, I. Fleming, B. Fisslthaler, et al., Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation, Nature 399 (6736) (1999) 601–605.
[20] D. Fulton, J.P. Gratton, T.J. McCabe, et al., Regulation of endothelium-derived nitric oxide production by the protein kinase Akt, Nature 399 (6736) (1999) 597–601.
[21] M.A. Medina, R. Munoz-Chapuli, A.R. Quesada, Challenges of antiangiogenic cancer therapy: trials and errors, and renewed hope, J. Cell Mol. Med. 11 (3) (2007) 374–382.
[22] A.R. Quesada, M.A. Medina, E. Alba, Playing only one instrument may be not enough: limitations and future of the antiangiogenic treatment of cancer, Bioessays 29 (11) (2007) 1159–1168.
[23] S.K. Mitra, D. Mikolon, J.E. Molina, et al., Intrinsic FAK activity and Y925 phosphorylation facilitate an angiogenic switch in tumors, Oncogene 25 (44) (2006) 5969–5984.
[24] I. Shiojima, K. Walsh, Role of Akt signaling in vascular homeostasis and angiogenesis, Circ. Res. 90 (12) (2002) 1243–1250.