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Apigenin suppresses the expression of VEGF, an important factor for angiogenesis, in endothelial cells via degradation of HIF-1α protein

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Abstract Apigenin, a plant-derived flavone, is a potent inhibitor of cell proliferation and angiogenesis, but the mechanisms leading to the pathological anti-angiogenic effects of apigenin are still unclear. In this study, we found that apigenin inhibited the hypoxia-induced expression of vascular endothelial growth factor (VEGF) mRNA in human umbilical artery endothelial cells. Apigenin also suppressed the expression of erythropoietin mRNA, which is a typical hypoxia-inducible gene, via the degradation of hypoxia-inducible factor 1 (HIF-1) α. We investigated the effect of apigenin on the interaction of HIF-1α with heat shock protein 90 (Hsp90), which is reported to be important for the stabilization of HIF-1α, and found that VEGF expression was inhibited via degradation of HIF-1α through interference with the function of Hsp90.

Keywords: Apigenin; Angiogenesis; Vascular endothelial growth factor; Hypoxia; Hypoxia-inducible factor; Heat shock protein 90

1. Introduction

Several polyphenolic compounds are recognized as cancer chemopreventive agents. Flavonoids are especially well known to suppress tumor cell growth via cell-cycle arrest and by the induction of apoptosis in several tumor cell lines [1–5]. Moreover, flavonoids inhibit endothelial cell proliferation and angiogenesis in vitro, the latter is endothelial cell cultures on collagen gels [6,7]. Angiogenesis is essential for the growth, progression, and metastasis of solid tumors [8]. Apigenin, a member of the flavone family that is present at high levels in many vegetables, has been found to inhibit ornithine decarboxylase and chemically induced skin tumorigenesis [9]. However, the mechanism by which apigenin suppresses angiogenesis has not been elucidated.

A prime regulator for angiogenesis is believed to be vascular endothelial growth factor (VEGF) and low oxygen tension dramatically induces the expression of this major angiogenic factor [10]. Transcriptional upregulation of VEGF has been implicated in the induction of genes; the induction is mediated by the specific binding of hypoxia-inducible factor 1 (HIF-1) to the hypoxia response element (HRE) [10–12]. The transcription factor HIF-1 is a heterodimer composed of HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT), also known as HIF-1β [13,14]. Although the ARNT protein is readily found in cells, HIF-1α is virtually undetectable in normal conditions. When cells are subjected to hypoxic conditions, the protein levels of the HIF-1α subunit increase rapidly. The existence of HIF-1 suggests that in the presence of oxygen, HIF-1α is regulated by two separate mechanisms: one involving prolyl hydroxylase, which initiates the degradation of HIF-1α, and another involving asparagine hydroxylase, which inactivates the C-terminal transactivation domain of HIF-1α [15]. Under normoxic conditions, the proteasome-dependent degradation of HIF-1α is mediated by prolyl hydroxylation, which permits the binding of the von Hippel–Lindau protein (pVHL), a component of the E3 ubiquitin ligase [16,17]. Under hypoxic conditions, prolyl hydroxylation of HIF-1α is blocked and the transcription factor HIF-1 is stabilized. Detailed study of HIF-1α protein revealed a 200-amino-acid sequence called the oxygen-dependent degradation (ODD) domain [18]. This domain is responsible for the degradation of HIF-1α in the presence of oxygen. pVHL mediates the ubiquitination and degradation of HIF-1α by binding to the ODD domain under normoxic conditions. It has been well documented that cobalt, a transition metal, mimics the effects of hypoxia by stabilizing HIF-1α [19]. However, the biochemical mechanisms underlying this stabilization differ from those underlying the stabilization induced by hypoxia. We have already reported that HIF-1α signal transduction during hypoxia was mediated by NADPH-P450 reductase (NPR) [20]. But NPR has little or no effect on erythropoietin (EPO) mRNA induction by cobalt. A recent study demonstrated that cobalt inhibits HIF–pVHL interaction even after hydroxylation of the proline residue [21], leading to a rapid accumulation of HIF-1α protein.

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Abbreviations: VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor 1; EPO, erythropoietin; pVHL, von Hippel–Lindau protein; Hsp90, heat shock protein 90; HRE, hypoxia response element; ARNT, aryl hydrocarbon receptor nuclear translocator; ODD, oxygen-dependent degradation; NPR, NADPH-P450 reductase; HUA-EC, human umbilical artery endothelial cells; RT-PCR, reverse-transcription PCR; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; PAS, Per-ARNT-Sim; bHLH, basic helix loop helix

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In this study, we tested the hypothesis that the anti-angiogenic effect of apigenin on tumor cells is caused by a reduction in the expression of VEGF, which is regulated by HIF-1 under hypoxic conditions. Moreover, we investigated a mechanism by which apigenin induces degradation of HIF-1α, independent of the ubiquitination of pVHL.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), EGM-2 medium, fetal calf serum, and *N*-CBZ-Leu-Leu-Norvalinal (proteasome inhibitor) were purchased from Sigma Chemical Co. (St. Louis, MO). Isogen was obtained from Nippon Gene (Toyama, Japan). An RNA PCR Kit (AMV) Ver. 2.1 was purchased from Takara (Shiga, Japan). Anti-human HIF-1α IgG and anti-human ARNT IgG were obtained from Novus (Littleton, CO) and Abcam (Cambridgeshire, UK), respectively. Anti-human heat shock protein 90 (Hsp90) IgG was purchased from BD Biosciences (San Jose, CA). Protein G–Sepharose 4 Faster Flow was supplied by Amersham Biosciences Corp. (Piscataway, NJ). Nitrocellulose membrane and 4-chloro-1-naphthol were purchased from Bio-Rad Laboratories (Hercules, CA). The Vectastain ABC kit (a biotin/avidin system) was obtained from Vector Laboratories (Burlingame, CA).

2.2. Cell culture

The human hepatoma cell line Hep3B was obtained from the Cell Resource Center for Biomedical Research at the Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). Human umbilical artery endothelial cells (HUA-EC) were obtained from Cambrex Bioscience Bio-Whittaker (Walkersville, MD). The Hep3B cells were maintained in DMEM containing 10% fetal calf serum and the HUA-EC in EGM-2 containing 10% fetal calf serum. To stimulate hypoxia, the cells were incubated in 5% O_2, 5% CO_2 and 90% N_2 balanced with a modulator incubator chamber (Napco 7101, Winchester, VA) or in a sealed 2.5-L box with an Anero Pack (oxygen absorber) for cells (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan).

2.3. Isolation of RNA and reverse-transcription PCR (RT-PCR)

With the use of Isogen, total RNA was extracted from Hep3B cells and from HUA-EC. The total RNA was transcribed into cDNA using the RNA PCR Kit according to the manufacturer's protocol. PCR with 10 pmol of each primer, 1.5 U of Ampli Taq, and 100 ng of cDNA was performed for 10 min at 94 °C and then 35 cycles of 1 min at 94 °C, 1 min at 56 °C and 2.5 min at 72 °C. The PCR primers for EPO [22] were 5′-GCCAGAGGAACTGACCAGAG-3′ (sense) and 5′-TTCTTCAGGTCATCCTATCC-3′ (antisense), while the PCR primers for VEGF [23] were 5′-TTCATGGATGTCTATCAGCG-3′ (sense) and 5′-CATCTCTCCTATGTGCTGGC-3′ (antisense). The oligonucleotide sequences of the reaction products were confirmed by sequencing.

2.4. Western blotting and immunoprecipitation

Cells at 80% confluence were lysed in 20 mM Tris–HCl buffer (pH 8.0) containing 0.1% Tween 20, 10% glycerol, 5 mM $MgCl_2$, 300 mM KCl, and 20 μM *N*-CBZ-Leu-Leu-Norvalinal. Cell lysates were divided into two and incubated with 2.8 μg of anti-HIF-1α IgG at 4 °C. Apigenin (100 μM) was added together with anti-HIF-1α IgG. After 2 h, 60 μL of protein G (10% vol/vol in lysis buffer) was added to each incubation mixture, and the incubation continued for another 2 h. Samples were washed twice by lysis buffer and protein G was removed by centrifugation. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) with 7.5% polyacrylamide gel. Using a previously described method [24], we performed immunoblotting with anti-ARNT IgG, anti-Hsp90 IgG, or antibodies against HIF-1α that were prepared in our laboratory [20]. Each protein was visualized using the ABC kit and 4-chloro-1-naphthol.

3. Results

3.1. Effect of apigenin on VEGF expression under hypoxia

HUA-EC were used to investigate the inhibitory effect of apigenin on VEGF expression (Fig. 1). When HUA-EC were exposed to 5% O_2 for 6 h, VEGF mRNA expression increased markedly. However, VEGF mRNA disappeared completely when apigenin (20 μM) was added to the culture medium. These results show that the flavone apigenin inhibited VEGF mRNA expression under hypoxic conditions.

3.2. Effect of apigenin on EPO expression during hypoxia

To examine whether or not apigenin inhibits the expression of EPO mRNA induced by hypoxia, we used human hepatocyte (Hep3B) cells and analyzed EPO mRNA expression by RT-PCR. The Hep3B cells were exposed to 5% O_2 for 3 h and then the expression of EPO mRNA was observed under hypoxic conditions (Fig. 2A). Apigenin dose-dependently reduced the expression. As HIF-1 was the transcription factor of the EPO gene during hypoxia, we studied the levels of HIF-1α and ARNT in Hep3B cells under hypoxic conditions by Western blotting (Fig. 2B). Although no immunoreactive band for HIF-1α was observed under normoxic conditions in Hep3B cells, HIF-1α was clearly expressed during hypoxia. When apigenin (100 μM) was added to the culture medium, the expression of HIF-1α was reduced. On the other hand, the levels of ARNT, a subunit of HIF-1, were not at all affected by treatment with apigenin.

3.3. Effect of apigenin on EPO expression induced by cobalt

Cobalt is known to inhibit the interaction between HIF-1α and pVHL directly and thereby prevent the degradation of HIF-1α [21]. To examine whether or not apigenin inhibits the cobalt-induced stabilization of HIF-1α, Hep3B cells were treated with apigenin in the presence of cobalt (Fig. 3A). Apigenin inhibited the expression of EPO mRNA induced by cobalt. Moreover, we tested the effect of apigenin on the levels of HIF-1α in Hep3B cells treated with cobalt (Fig. 3B). HIF-1α expression increased on treatment with cobalt, whereas it apparently decreased when apigenin (100 μM) was added to the culture medium. These results indicate that apigenin inhibits the stabilization of HIF-1α in the presence of cobalt. The presence of apigenin did not change the ARNT levels. When apigenin was present, HIF-1α was degraded under hypoxic conditions or in the presence of cobalt ion under normoxic conditions, suggesting that apigenin degraded HIF-1α via a pVHL-independent pathway.

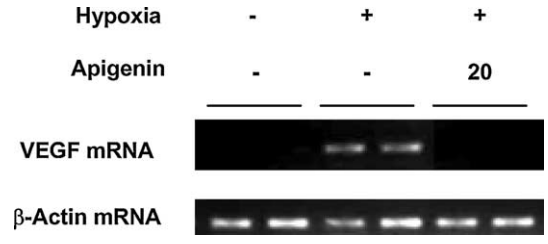

Fig. 1. Expression of VEGF mRNA in HUA-EC. HUA-EC were exposed to 5% O_2, 5% CO_2 and 90% N_2 for 6 h in the presence or absence of 20 μM apigenin. VEGF mRNA expression was analyzed by RT-PCR.

3.4. Hsp90 is required for the stabilization of HIF-1α during hypoxia

Using geldanamycin, a specific inhibitor of Hsp90, Minet et al. [25] demonstrated that Hsp90 was essential for stabilizing HIF-1. In addition, HIF-1α belongs to the Per-ARNT-Sim (PAS)-basic helix loop helix (bHLH) family and interacts with Hsp90 [25,26]. We hypothesized that the degradation of HIF-1α by apigenin correlates with the interaction between HIF-1α and Hsp90. We prepared cell lysates from Hep3B under conditions of hypoxia in the absence of apigenin. Apigenin was added to the lysates together with anti-HIF-1α IgG and then the complex anti-HIF-1α IgG was collected using protein G. The samples were analyzed by SDS–PAGE with 7.5% polyacrylamide gel. The antibody against HIF-1α could pull down Hsp90 protein, indicating that Hsp90 is associated with HIF-1α (Fig. 4). When apigenin was added to the cell lysates together with the antibody, the band of Hsp90 disappeared, indicating that apigenin inhibited the interaction of HIF-1α with Hsp90. Geldanamycin also reduced the levels of EPO mRNA in Hep3B cells under hypoxic conditions (data not shown).

4. Discussion

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 [27]. Several cancers, including breast 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. Researchers have identified the isoflavonoid genistein as a potent inhibitor of angiogenesis in vitro [6,7]. A recent study demonstrated that the anti-angiogenic effect of genistein was due to inhibition of HIF-1, an important regulator of VEGF gene homeostasis particularly under low-oxygen conditions [28]. The flavone apigenin has also been proposed as an antitumor agent [29]. However, the molecular mechanisms involved in the anti-angiogenic effects of apigenin are not well understood. This study investigated the anti-angiogenic effects of apigenin on cells under hypoxic conditions. We observed that apigenin suppressed the expression of both VEGF mRNA and EPO mRNA induced by hypoxia in HUA-EC and in Hep3B cells. Apigenin proved more effective against the expression of VEGF mRNA and EPO mRNA than genistein (data not shown). In this study, we found that apigenin caused the degradation of HIF-1α but not of ARNT. Given that genistein has been reported to inhibit HIF-1 DNA-binding activity [28], apigenin and genistein may have different angiogenic mechanisms.

Apigenin inhibited the mRNA expression of EPO induced by cobalt, which mimics hypoxia; it also inhibited the stabilization of HIF-1α induced by cobalt. Because the mechanism underlying the stabilization of HIF-1α during hypoxia is known to differ from that induced by cobalt, we speculate that the degradation of HIF-1α by apigenin is independent of the ubiquitination by pVHL. As geldanamycin reduced the expression of EPO mRNA in Hep3B cells under hypoxic conditions (data not shown), we examined the effect of apigenin on the interaction with HIF-1α of the chaperone protein Hsp90, which associates with HIF-1α to stabilize an activated form of HIF-1 during hypoxia [25,30]. Through immunoprecipitation experiments, we found that apigenin disturbed the binding of Hsp90 to HIF-1α. These results indicate that apigenin diminishes the hypoxia-induced interaction of HIF-1α with Hsp90 to release HIF-1α, which in turn undergoes degradation.

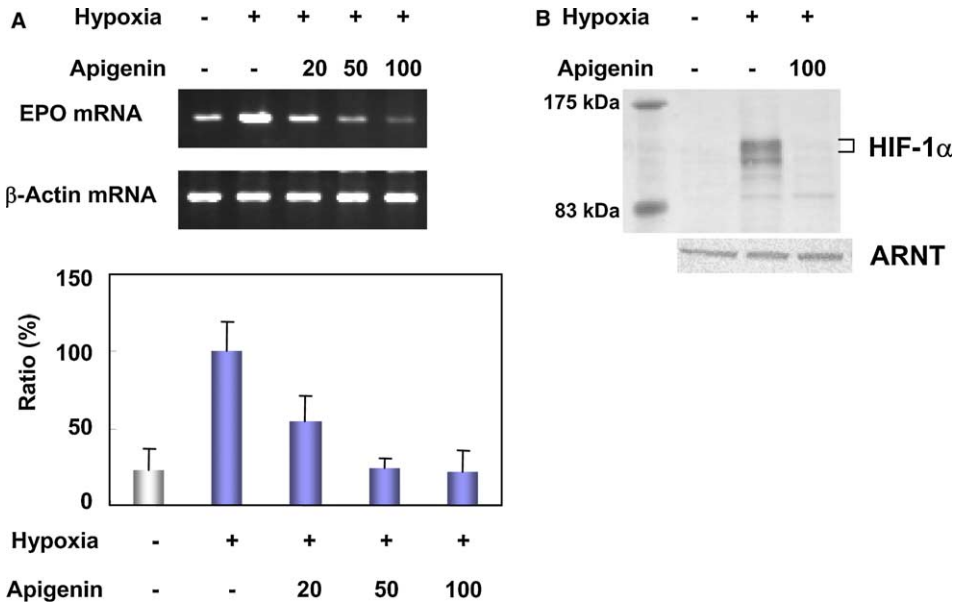

Fig. 2. Expression of EPO mRNA and HIF-1α in Hep3B cells under hypoxic conditions. (A) Hep3B cells were exposed to 5% O_2, 5% CO_2 and 90% N_2 for 3 h in the presence or absence of 20, 50, or 100 μM apigenin. EPO mRNA expression was analyzed by RT-PCR. The ratio (EPO mRNA/β-Actin mRNA) under hypoxia was 100%. Values are given as means ± S.D. for four separate experiments. (B) Hep3B cells were exposed to 5% O_2, 5% CO_2 and 90% N_2 for 3 hours in the presence or absence of 100 μM apigenin. Whole cell lysates were immunoprecipitated with anti-HIF-1α IgG and analyzed by SDS–PAGE with 7.5% poly-acrylamide gel, and then were immunoblotted with antibodies against HIF-1α. Whole cell lysates (50 μg) were analyzed by SDS–PAGE with 7.5% polyacrylamide gel and immunoblotted with anti-ARNT IgG. An ABC kit was used for the secondary antibody and HIF-1α was detected with 4-chloro-1-naphthol.

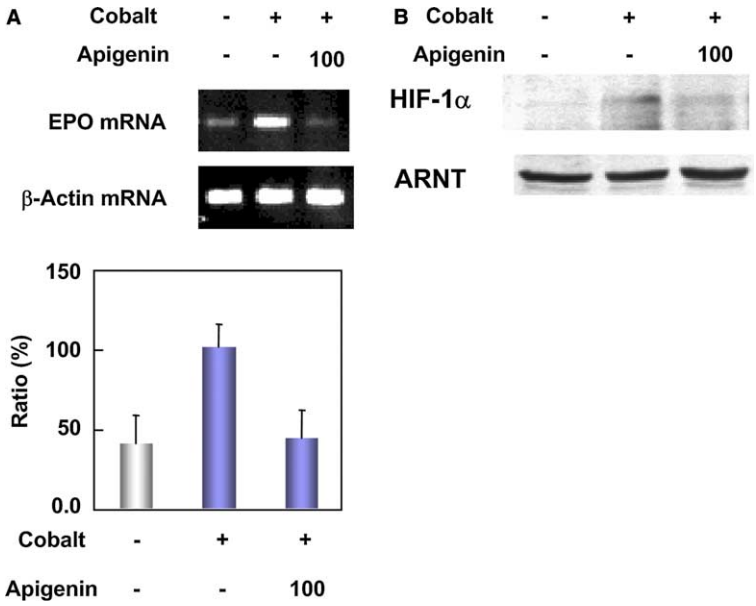

Fig. 3. Expression of EPO mRNA and HIF-1α in Hep3B cells treated with cobalt. Hep3B cells were exposed to 100 μM cobalt for 3 h in the presence or absence of 100 μM apigenin. (A) EPO mRNA expression was analyzed by RT-PCR. The ratio (EPO mRNA/β-Actin mRNA) under hypoxia was 100%. Values are given as means ± S.D. for four separate experiments. (B) Whole cell lysates were analyzed by SDS–PAGE with 7.5% poly-acrylamide gel and immunoblotted with antibodies against HIF-1α (1:100). An ABC kit was used for the secondary antibody and HIF-1α was detected with 4-chloro-1-naphthol.

We studied whether or not flavonoids other than apigenin have an inhibitory effect on the expression of EPO mRNA induced by hypoxia. We found that kaempferol and myricetin have an inhibitory effect on the expression under hypoxic conditions, but catechin and epicatechin do not (data not shown). Wilson et al. [31] demonstrated that the flavonoid quercetin stabilizes HIF-1α and induces the expression of VEGF mRNA under normoxic conditions. Moreover, it has been proposed that quercetin regulates the response to hypoxia by inhibiting the activity of HIF hydroxylase, a member of the Fe(II), 2-oxoglutarate-dependent dioxygenase family, because quercetin is a good iron chelator [32].

In this study, we found that apigenin reduced HIF-1α levels by interfering with the binding of HIF-1α to Hsp90, leading to a suppression of EPO mRNA transcription. The angiogenesis frequently observed in tumors would be suppressed by apigenin via a reduction of VEGF mRNA expression. Since it may suppress a number of genes induced by HIF-1α, it is likely that apigenin could be a potent inhibitor of angiogenesis. Our results clearly show that apigenin is potentially an important chemical for controlling HIF-1α levels.

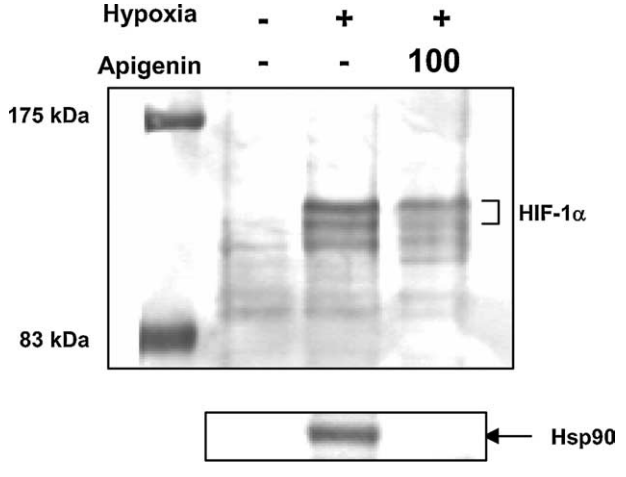

Fig. 4. Interaction of HIF-1α and Hsp90 proteins in the presence of apigenin. Hep3B cells were exposed to 5% O_2, 5% CO_2 and 90% N_2 for 3 h. Apigenin (100 μM) was added to whole cell lysates. The lysates were immunoprecipitated with anti-HIF-1α IgG and analyzed by SDS–PAGE with 7.5% polyacrylamide gel and immunoblotted with antibodies against HIF-1α (1:100) (A) and anti-Hsp90 IgG (B).

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