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Flavonoids inhibit hypoxia-induced vascular endothelial growth factor expression by a HIF-1 independent mechanism

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ABSTRACT

Flavonoids are a group of polyphenolic dietary compounds that have been proposed to possess chemopreventive properties against lung cancer. In this work we analyzed the effect of a group of 20 structurally related flavonoids, including flavones, flavonols and isoflavones, on the production of vascular endothelial growth factor (VEGF) induced by hypoxia in NCI-H157 cells. VEGF is the main regulator of physiological and pathological angiogenesis and is highly stimulated by hypoxia-inducible factor 1 (HIF-1). We found that apigenin, luteolin, fisetin and quercetin inhibited hypoxia-induced VEGF expression in the low micromolar range. Structure–activity relationships demonstrated that flavone derivatives were the most active compounds and that hydroxylation of the A ring at the positions 5 and 7 and of the B ring at the 4′ position were important for this activity. Interestingly, only a group of VEGF inhibitors, including apigenin, flavone and 4′,7-dihydroxiflavone, reduced the expression of HIF-1α under these conditions, whereas others, such as fisetin, luteolin, galangin or quercetin, induced HIF-1α expression while reducing those of VEGF. When cells were exposed to hypoxia in the presence of these flavonoids, HIF-1α translocated to the nucleus and interacted with p300/CBP, but this complex was transcriptionally inactive. Taken together these findings indicate that flavonoids impair VEGF transcription by an alternative mechanism that did not depend on nuclear HIF levels. We also found that flavonoids suppressed hypoxia-induced STAT3 tyrosine phosphorylation and that this activity correlated with their potency as VEGF inhibitors, suggesting that inhibition of STAT3 function may play a role in this process.

1. Introduction

Flavonoids are a family of polyphenolic compounds that are common components of the human diet. They have been studied as potential anticarcinogens because flavonoids stimulate apoptosis, inhibit proliferation and show antioxidant activity [1]. Recent data indicate that some of them can also inhibit vascular endothelial growth factor (VEGF) expression [2–9] and tumor angiogenesis *in vivo* [4,6,10,11]. VEGF is highly stimulated by hypoxia-inducible factor 1 (HIF-1), a transcription factor that consists of two subunits, HIF-1α and HIF-1β [12]. In normoxic conditions HIF-1α is hydroxylated by prolyl hydroxylases at two specific prolyl residues located in the oxygen degradation domain. These modifications allow the binding of HIF-1α to the von Hippel–Lindau (pVHL) tumor suppressor protein which catalyzes HIF-1α ubiquitination, targeting it for proteasomal degradation. Under hypoxia prolyl hydroxylases are inhibited, HIF-1α translocates to the nucleus and dimerizes with the constitutively expressed HIF-1β [13]. Once imported into the nucleus, the heterodimeric HIF-1 factor binds to hypoxia-responsive elements (HREs) located within the promoter of a battery of genes related to the adaptative response to hypoxia [14]. Transcriptional activity of HIF-1 requires the additional interaction of HIF-1 with the coactivators p300 and cAMP-response element-binding protein (CBP) [12]. In the presence of oxygen the ability of HIF-1α to activate transcription is also repressed, because the hydroxylation of an asparagine residue in the C-terminal transactivation domain abrogates its interaction with p300 [15]. Signal transducer and activator of transcription 3 (STAT3) has also been shown to mediate VEGF transcription. Binding of both STAT3 and HIF-1α to the VEGF promoter is

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required for maximum transcription of VEGF following hypoxia, and loss of binding by either transcription factor severely compromises VEGF expression [16]. More recently, it has been proposed that FOXO3a may act as a negative regulator of HIF-1 transcriptional activity by interfering with the ability of p300 to function as a transcriptional coactivator [17].

HIF-1α can also be induced in normoxia by growth factors and oncogenic signaling [12]. Non-hypoxic activation of HIF-1α is achieved through major signal transduction pathways, including phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which is required for cap-dependent HIF-1α mRNA translation, and mitogen-activated protein kinase pathway (MAPK) that phosphorylates HIF-1α promoting its nuclear accumulation and transcriptional activity [12,18]. HIF-1α expression is also regulated by other proteins that modulate protein degradation, such as p53/Mdm2, Hsp90 or RACK1 [19–21]

It seems that more advanced stage tumors actually express higher levels of VEGF protein. For example, high expression levels of VEGF mRNA and protein in lung cancer patients correlated with poor prognosis, indicating that advanced lung cancer tissues tend to express VEGF [22]. Epidemiological studies suggest that flavonoids may have chemopreventive properties against several types of cancer, particularly lung cancer [23–25]. Several flavonoids inhibit HIF-1α and/or VEGF expression in tumoral cells including apigenin [2–4,6,9], genistein [11] and chrysin [7]. In addition, luteolin has been reported to inhibit VEGF-mediated angiogenic signalling [10]. However, the structural basis and the molecular pathways implicated in the inhibition of VEGF expression induced by hypoxia have not been fully clarified. Nowadays it is assumed that flavonoids affect VEGF expression by reducing HIF-1α intracellular protein levels. In this work, we analyzed the effect of 20 structurally related flavonoids on hypoxia-induced VEGF expression in non-small cell lung carcinoma H157 cell line. We found that flavone derivatives were the most active compounds and that substitution of two hydroxyl groups in the A ring and the presence of an additional hydroxyl group at 4′ of the B ring were important for this activity. We also demonstrated that active flavonoids impaired VEGF transcription by a mechanism that did not depend on nuclear HIF levels, and suggest that the inhibition of STAT3 phosphorylation induced by hypoxia may play a role in this process.

2. Materials and methods

2.1. Reagents and antibodies

Flavonoids, resveratrol and wortmannin were purchased from Sigma (St. Louis, MO, USA), dissolved in dimethylsulfoxide (DMSO) and added to the medium at the indicated concentrations, maintaining DMSO concentrations below 0.5%. The antibodies against Akt, phospho-Akt (Ser ^{473}), histone 3, acetylated histone 3 (Lys^{23}), FOXO3a, phospho-FOXO3a (Ser^{253}), phospho-ERK1/2 (Thr^{202}/Tyr^{204}), STAT3 and phospho-STAT3 (Tyr^{705}) were from Cell Signaling Technology (Boston, MA, USA). Anti-VEGF antibody (MAB293) was from R&D (Minneapolis, MN, USA). HIF-1α and p300 antibodies were purchased from BD Biosciences (Erembodegem, Belgium) and β-actin antibody was purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture

The lung cancer squamous cell carcinoma NCI-H157 cell line was purchased from Promochem SL (Barcelona, Spain) and cultured in RPMI 1640 medium with Ultraglutamine 1 (BioWhittaker, Verviers, Belgium) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium). Cells were grown in a humidified atmosphere containing 5% CO_2 at 37 °C. Microenvironmental hypoxic conditions (1% O_2) were achieved in an airtight humidified chamber infused with a mixture of 1% O_2, 5% CO_2 and 94% N_2 (Air Liquide, Madrid, Spain). The chamber was monitored with a O_2 sensor (Dräger Pac 5000, Dräger, Lübeck, Germany) and temperature was maintained at 37 °C.

2.3. Western blotting

5×10^6 H157 cells were plated on 100 mm culture dishes in complete medium for 12 h. Cells were exposed to medium without serum for 2 h and then pretreated with the indicated concentrations of each flavonoid for 30 min, followed by exposure to hypoxia for 6 h. When necessary, nuclear and cytoplasmic extracts were separated using the NE-PER kit following the manufacturer's instructions (Pierce, Rockford, IL, USA). Total cellular extracts were obtained by homogenizing the cells at 4 °C in 20 mM Tris–HCl (pH 8.0), 0.4 mM Na_3VO_4, 1% SDS and 1× Complete mini protease inhibitor cocktail tablets (Roche Diagnostics, Basel, Switzerland). Samples were treated with Laemmli sample buffer, separated by SDS-PAGE under reducing conditions and transferred to a nitrocellulose membrane (Protran, Schleicher & Schuell Bioscience, Dassel, Germany). Membranes were blocked with 10% nonfat milk in TBS containing 0.1% Tween-20 for 1 h, and then incubated overnight at 4 °C with the primary antibody at the suitable dilution. Blots were incubated with a HRP-conjugated secondary antibody for 1 h, and developed with Lumi-Light Plus western blotting substrate (Roche, Mannheim, Germany). Blot images were scanned with a GS-800 calibrated densitometer and analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA).

2.4. Screening assay to measure the effect of flavonoids on VEGF production in H157 cells

4×10^4 H157 cells were plated in 96-well plates and cultured until confluence was reached. Medium was then removed and cells were washed and incubated with different concentrations of each flavonoid in 200 μl of serum-depleted medium under normoxia or hypoxia for 24 h. Triplicates were used for each experimental condition. Supernatants (100 μl) were added to a 96-well bio-dot microfiltration apparatus (Bio-Rad, Hercules, CA, USA) with a multichannel pipette, filtered under vacuum through a Protran nitrocellulose membrane and washed twice with 100 μl of TBS. The membrane was then blocked with 10% nonfat milk in TBS containing 0.1% Tween-20 for 1 h, and VEGF was immunodetected with MAB293 anti-VEGF antibody (R&D, Minneapolis, MN, USA) and quantified as before. Dose–response curves were obtained by nonlinear regression of the original data using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

VEGF secretion into the culture medium was also measured by western blot under non-reducing conditions with the same antibody following the manufacturer's instructions (R&D, Minneapolis, MN, USA). Briefly, cells were grown in P-100 plates in complete medium, washed twice with PBS, and incubated in medium without serum under normoxia (21% O_2) or hypoxia (1% O_2) for 24 h. Supernatants were then collected, concentrated with a centrifugal filter device (Millipore, Bedford, MA, USA), and the amount of VEGF was measured by western blot under non-reducing conditions.

2.5. Cell viability and cell proliferation assays

For cell viability assays, 4×10^4 H157 cells per well were seeded in 96-well plates (TPP, St. Louis, MO, USA) in complete medium and incubated for 12 h before experimental treatments. Then cells

were treated in triplicate with flavonoids in medium without serum containing 25 mM HEPES and L-glutamine (GIBCO, Invitrogen, Carlsbad, CA, USA) and further incubated in normoxia (21% O_2) or hypoxia (1% O_2) for 24 h. After this time medium was removed and viable cells measured using the neutral red assay [26]. Conventional MTT tetrazolium reduction assay was not suitable for these experiments since some flavonoids directly reduce MTT in the absence of living cells [27]. Neutral red assay was performed by incubating the cells for 90 min at 37 °C with 200 μl of fresh media, supplemented with 45 mM HEPES, to avoid neutral red precipitation, and 50 μl of a solution prepared by diluting 1 mg/ml neutral red (SIGMA Chemical, St. Louis, MO, USA) with an equal volume of 1.8% NaCl solution. Then cells were washed twice with PBS and lysed with 100 μl of NaH_2PO_4–ethanol 50%. The absorbance of the extracted dye was read at 540 nm in a Multiskan Ex plate reader (Thermo Fisher Scientific, Vantaa, Finland). Proliferation assays were performed as described [26]. Briefly 3000 H157 cells/well were plated and then treated with flavonoids prepared in complete medium containing 25 mM HEPES for 72 h in normoxic conditions. Then medium was removed and the number of cells quantified.

2.6. Semi-quantitative reverse transcription-PCR analysis

Total RNA was isolated from H157 cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Two micrograms from each RNA sample was reverse-transcribed into cDNA using the Superscript II reverse transcriptase (Promega, Madison, WI, USA) in a final reaction volume of 20 μl. Gene amplification was done as described [26] using the following primers: VEGF-A: forward 5′-GCCTCGGCTTGTCACAT-3′ and reverse 5′-GAGTGCCCACTGAGGAGTC-3′, GLUT-3: forward 5′-AGGCTCGATGCTGTTCATCT-3′ and reverse 5′-ACCGGCTTCCTCATTACCTT-3′ and β-actin: forward, 5′-GGTGAGGATCTTCATGAGGT-3′ and reverse, 5′-TCTACAATGAGGTGCGTGTG-3′.

2.7. Immunoprecipitation analysis

For immunoprecipitation analyses 5×10^6 H157 cells were plated on 100 mm culture dishes in complete medium for 12 h. Then medium was removed, and cells were treated with 20 μM of the indicated flavonoid in medium without serum containing 25 mM HEPES and further incubated in normoxia (21% O_2) or hypoxia (1% O_2) for 6 h. Cells were then immediately treated on ice for 30 min with lysis buffer (50 mM Tris–HCl pH 7.5, 1% Triton-X100, 50 mM NaF, 5 mM EDTA, 0.1 mM Na_3VO_4, 100 μM deferoxamine, 500 mM NaCl and 1× Complete mini protease inhibitor cocktail tablets), followed by two freeze–thaw vortex cycles. Protein concentration was measured in the supernatant using BCA protein assay kit (Pierce, Thermo Scientific, Rockfork, IL, USA) and 500 μg of total proteins were incubated with 1 μg of anti-human p300 antibody in a final volume of 500 μl for 1 h at 4 °C on a rotating device. Then, 20 μl of protein A/G plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and samples were incubated overnight at 4 °C on a rotating device. Samples were washed 3 times with 1 ml lysis buffer and pellets were resuspended in 20 μl of 1× electrophoresis sample buffer. Samples were boiled for 7 min, centrifuged and the supernatant analyzed by western blot with the specified antibody.

3. Results

3.1. Flavonoids inhibit hypoxia-induced VEGF expression in H157 cells

Our first goal was to set-up a screening assay to measure the effect of a large number of compounds on the secretion of VEGF induced by hypoxia by lung tumor cells. For this purpose we included a variety of cell lines derived from small cell lung cancer (N417 and H69) and non-small cell lung cancer (A549, H157 and H1299). H157 cells secreted the highest amount of VEGF under hypoxic conditions, while expression in A549 was barely detectable (Fig. 1A). N417 cells also produced high amounts of VEGF in response to hypoxia. Nevertheless, non-small cell lung cancer represents 75–80% of all primary cancers of the lung and consequently the H157 cell line was selected for the screening. This screening was carried out in 96-well plates that underwent hypoxia (1% O_2) for 24 h. Supernatants were collected, filtered through a nitrocellulose membrane using a dot-blot apparatus and VEGF was immunodetected with a suitable antibody (Fig. 1B). The viability of cells cultured under hypoxia or normoxia with each flavonoid was simultaneously measured in 96-well plates. Using this scheme, we tested the activity of 20 structurally related flavonoid derivatives (see structures in Table 1) using resveratrol, a natural polyphenol that has been reported to inhibit both HIF-1α and VEGF expression under hypoxia [5], as a positive control (Fig. 1C).

As shown in Table 2, luteolin, fisetin, apigenin and quercetin were the most effective VEGF inhibitors, with IC_{50} values around 5–8 μM. A second group of somewhat less active compounds included chrysin, genistein, 4′,7-dihydroxyflavone and kaempferol, that showed IC_{50} values ranging from 11 to 16 μM. Flavone and resveratrol were active at higher concentrations (IC_{50} above 20 μM). Galangin, 3,7-dihydroxyflavone and formononetin barely affected VEGF production, while biochanin 5-hydroxyflavone, 7-hydroxyflavone, naringenin, equol and taxifolin were inactive in this assay. A noteworthy fact is that myricetin increased VEGF production over hypoxia-induced levels that might be related to the presence of a third hydroxyl group at the 5′-position in the B ring (Table 2).

As judged by their effects on cellular viability and cell proliferation, flavonoids were more cytostatic than cytotoxic (Table 2). In any case, important differences in cytotoxicity were found between VEGF inhibitors. Fisetin and luteolin affected cell viability with IC_{50} values around 20 μM, and quercetin showed cytotoxic effects below 50 μM. On the other hand, apigenin, that inhibited hypoxia-induced VEGF as effectively as fisetin or luteolin, did not show cytotoxicity at concentrations below 100 μM. No differences in cell viability under normoxia or hypoxia were found with any of the flavonoids tested.

3.2. Flavonoids inhibited VEGF expression irrespective of HIF-1α levels under hypoxic conditions

Several studies have proposed that flavonoids downregulate HIF-1α protein levels, and therefore the expression of its downstream genes, through the inhibition of the PI3K/Akt pathway. For this reason we measured the effect of flavonoids on Akt phosphorylation and HIF overexpression in response to hypoxia. To determine the optimal treatment period, HIF-1α expression was measured at 1, 2, 4, 6, 8, 12 and 24 h after hypoxic exposure of H157 cells. HIF-1α peaked at 4–6 h and then slowly decreased, as found in most cellular lines where it has been measured (data not show). For this reason we selected 6 h as the treatment period. As expected, wortmannin, a selective inhibitor of PI3-kinase, completely blocked Akt phosphorylation and reduced HIF-1α expression (Fig. 2). Similarly, resveratrol an apigenin inhibited Akt phosphorylation and decreased hypoxia-induced HIF-1α protein levels, as described in other cellular types [3,5,9]. Surprisingly, three of the four best inhibitors of VEGF expression, luteolin, fisetin and quercetin that also inhibited Akt phosphorylation, induced HIF-1α expression under hypoxic conditions, as did galangin (Fig. 2B), suggesting that these molecules inhibited VEGF

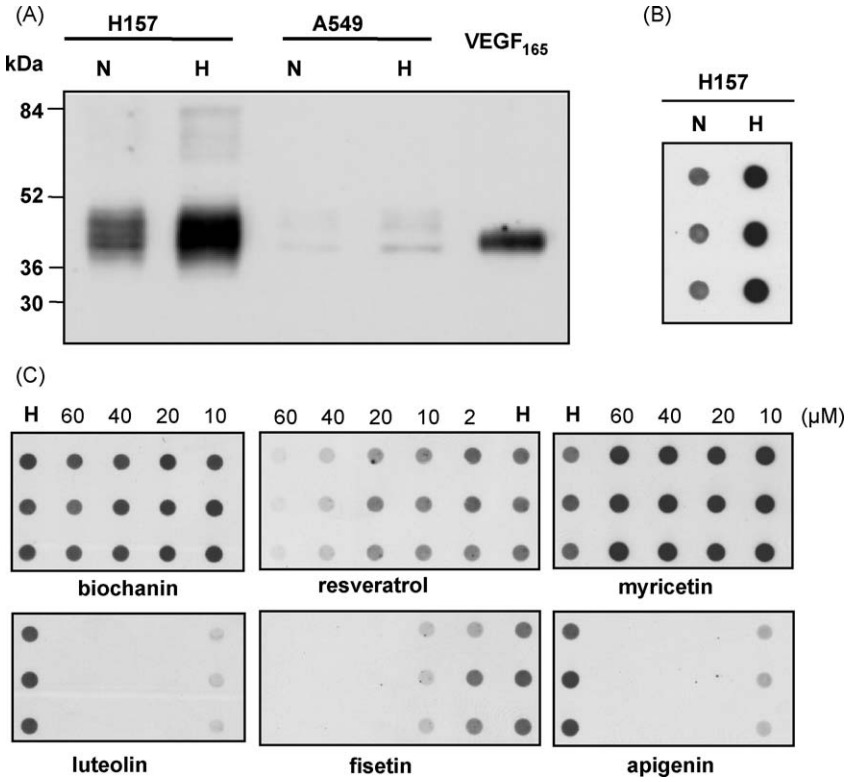


Fig. 1. Flavonoids inhibited hypoxia-induced VEGF production in H157 cells. (A) Up-regulation of VEGF expression by hypoxia. H157 and A549 cells were grown in P-100 plates in complete medium, and then incubated in medium without serum under normoxia (21% O_2) or hypoxia (1% O_2) for 24 h. After this time supernatants were collected, concentrated with a centrifugal filter device (Millipore), and the amount of VEGF was measured by western blot under non-reducing conditions. The isoform detected corresponds to VEGF-165, as judged by the mobility of the recombinant protein used as a control. (B) Screening assay to measure the production of VEGF from H157 cells by dot-blot. Cells were plated in 96-well plates, incubated under hypoxia (H) or normoxia (N) for 24 h in medium without serum, and 100 μl of each supernatant were filtered through a nitrocellulose membrane in a dot-blot apparatus. VEGF was immunodetected as before. Triplicates were made of each condition. (C) Effect of flavonoids on the production of VEGF from H157 cells incubated under hypoxia as measured by dot-blot. Luteolin, fisetin and apigenin inhibited VEGF production in a dose–response manner, while myricetin stimulated VEGF production under these conditions. Biochanin and resveratrol were used as negative and positive controls, respectively.

expression by a mechanism that did not depend on total HIF-1α protein.

For this reason we tested whether this overexpressed HIF-1α was functionally active by measuring the effect of these flavonoids on the transcription of two HIF-1 target genes: VEGF and GLUT3. Expression of VEGF mRNA was abolished by 40 μM fisetin, apigenin and luteolin, and to a lesser extent by quercetin (Fig. 3). It is interesting to note that these compounds reduced VEGF mRNA levels below those seen in normoxia, as found with VEGF protein. Other molecules such as quercetin and galangin also inhibited VEGF transcription, but to a lower extent (Fig. 3B). Similar effects were seen when expression of GLUT3 was analyzed, though inhibitions reached were lower than in the case of VEGF (Fig. 3B).

It has been recently reported that some flavonoids that induce HIF-1α under normoxic conditions may block its nuclear translocation, impairing in this way the transcriptional activity of this factor [28]. To test this possibility we isolated cytoplasmic and nuclear protein extracts after treating the cells with these flavonoids under hypoxic conditions and measured HIF-1α localization by western blot. As seen in Fig. 4A, galangin, quercetin or fisetin not only induced total HIF-1α expression, but strongly increased the nuclear concentration of this factor. These results show that inhibition of HIF1α translocation by these flavonoids was not responsible for the impaired transcriptional activity of HIF-1α under hypoxia.

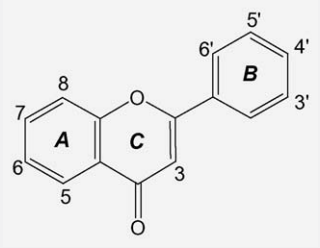
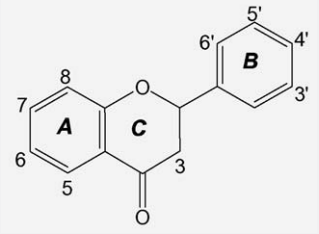
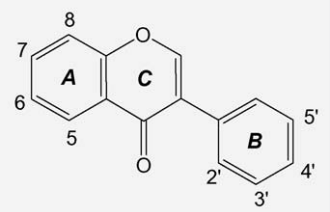
3.3. Flavonoids did not block p300/HIF-1α binding in H157 cells

It has been described that nuclear FOXO3a negatively regulates HIF-1 transcriptional activity by impairing p300/HIF-1α interaction [17]. For this reason we studied whether inhibition by flavonoids of FOXO3a phosphorylation could mediate its nuclear accumulation and the subsequent inactivation of HIF-1. Fig. 4B shows that, as expected, apigenin, luteolin and quercetin, that inhibited Akt phosphorylation, reduced phospho-FOXO3a in the cytoplasm. However, total FOXO3a did not increased into the nucleus as a consequence of this inhibition, as described in other cell lines when the PI3K/Akt pathway was blocked [29,30]. Treatment with 0.2 μM wortmannin was also ineffective at inducing FOXO3a nuclear localization in H157 cells under these conditions.

To test whether flavonoids impaired the transcriptional activity of HIF-1α by affecting its interaction with p300, H157 cells were incubated with flavonoids under hypoxia and HIF-1α/p300 complex was immunoprecipitated with an antibody against p300. As shown in Fig. 5, compounds that reduced the expression of HIF-1α, such as flavone or apigenin, also reduced the amount of HIF bound to p300. On the other hand, flavonoids such as fisetin, galangin, quercetin, myricetin or kaempferol, that stimulated HIF-1α under hypoxic conditions, also raised the amount of HIF-1 bound to p300. These results show that inhibition of the interaction of p300 with HIF-1 did not account for the inhibition of VEGF expression found with these flavonoids.

In the search of other factors that may be mediating the transcriptional regulation of VEGF by flavonoids we tested whether flavonoids affected histone acetylation. It has been described that histone deacetylase inhibitors repress the transactivation potential of HIF without affecting HIF-1α protein levels [31]. We measured whether flavonoids induced the acetylation of histone 3 using trichostatin A (TSA), a non-specific histone

Table 1
Structure of the flavonoids used in this study.

Flavones and flavonols	Flavanones	Isoflavones			
					
	3	5	7	3′	4′	5′
Flavones						
Flavone						
5-Hydroxyflavone		OH				
7-Hydroxyflavone			OH			
3,7-Dihydroxyflavone	OH		OH			
Chrysin		OH	OH			
4′,7-Dihydroxyflavone			OH		OH	
Apigenin		OH	OH		OH	
Luteolin		OH	OH	OH	OH	
Fisetin	OH		OH	OH	OH	
Flavonols						
Galangin	OH	OH	OH			
Kaempferol	OH	OH	OH		OH	
Quercetin	OH	OH	OH	OH	OH	
Myricetin	OH	OH	OH	OH	OH	OH
Isoflavones						
Daidzein			OH		OH	
Formononetin			OH		OCH_3	
Genistein		OH	OH		OH	
Biochanin		OH	OH		OCH_3	
Equol[a]			OH		OH	
Flavanones						
Naringenin		OH	OH		OH	
Taxifolin	OH	OH	OH	OH	OH	

[a] Equol: 4′,7-isoflavandiol.

Table 2
Effect of flavonoids on hypoxia-induced VEGF secretion, cell viability and cell proliferation in H157 cells[a].

	$IC_{50} \pm$ S.D. (μM)			
	VEGF	Cell viability		Cell proliferation
		Hypoxia	Normoxia	
Luteolin	5.5 ± 0.2	21 ± 3	19 ± 5	13 ± 2
Fisetin	5.5 ± 0.7	21 ± 4	28 ± 2	26 ± 1
Apigenin	5.9 ± 0.1	>100	>100	35 ± 4
Quercetin	8.5 ± 1.9	48 ± 8	45 ± 6	33 ± 3
Chrysin	11 ± 2	>100	>100	47 ± 2
Genistein	14 ± 1	100 ± 6	>100	48 ± 5
4′,7-Dihydroxyflavone	15 ± 2	>100	>100	62 ± 6
Kaempferol	16 ± 1	96 ± 21	82 ± 10	61 ± 3
Flavone	23 ± 2	>100	>100	88 ± 13
Resveratrol	25 ± 3	104 ± 3	100 ± 2	57 ± 6
3,7-Dihydroxyflavone	45 ± 6	>100	>100	29 ± 3
Galangin	71 ± 5	>100	>100	48 ± 1
Formononetin	77 ± 5	>100	>100	>100
Biochanin	>100	>100	>100	73 ± 6
Myricetin	stimulated[b]	>100	>100	20 ± 3

[a] Inhibition of hypoxia-induced VEGF expression, cell viability and cell proliferation were measured as described in Materials and Methods. Note that cell viability was measured in cells cultured in medium without serum.

[b] Myricetin stimulated VEGF expression by 2-fold at 40 μM and by 1.7-fold at 20 μM with respect to control cells exposed to hypoxia.

deacetylase inhibitor, as a positive control. TSA inhibited hypoxia-induced VEGF production in H157 cells with a IC_{50} of 0.2 ± 0.05 μM. At this concentration TSA strongly stimulated histone-3 acetylation as measured by western blot (>10-fold). However, when flavonoids were tested at 80 and 10 μM in this assay no changes in histone 3 acetylation were detected (data not shown).

3.4. Flavonoids inhibit hypoxia-induced STAT3 activation

Signal transducer and activator of transcription 3 (STAT3) has also been shown to mediate VEGF transcription. Activation of STAT3 by tyrosine phosphorylation leads to its dimerization and translocation to the nucleus. HIF-1α and STAT3 can bind simultaneously to the VEGF promoter and both are required for maximum transcription of VEGF mRNA following hypoxia [16]. For this reason we tested whether flavonoids were affecting STAT3 activation mediated by hypoxia. Fig. 5B shows that flavonoids inhibited STAT3 phosphorylation following exposure to hypoxia. Interestingly, their potency as STAT3 inhibitors correlated with their potency as VEGF inhibitors. For example, luteolin and quercetin at 40 and 10 μM decreased the phosphorylation of STAT3 to an undetectable level. On the other hand apigenin, fisetin and galangin showed this activity at 40 μM, while resveratrol and wortmannin were inactive in this assay.

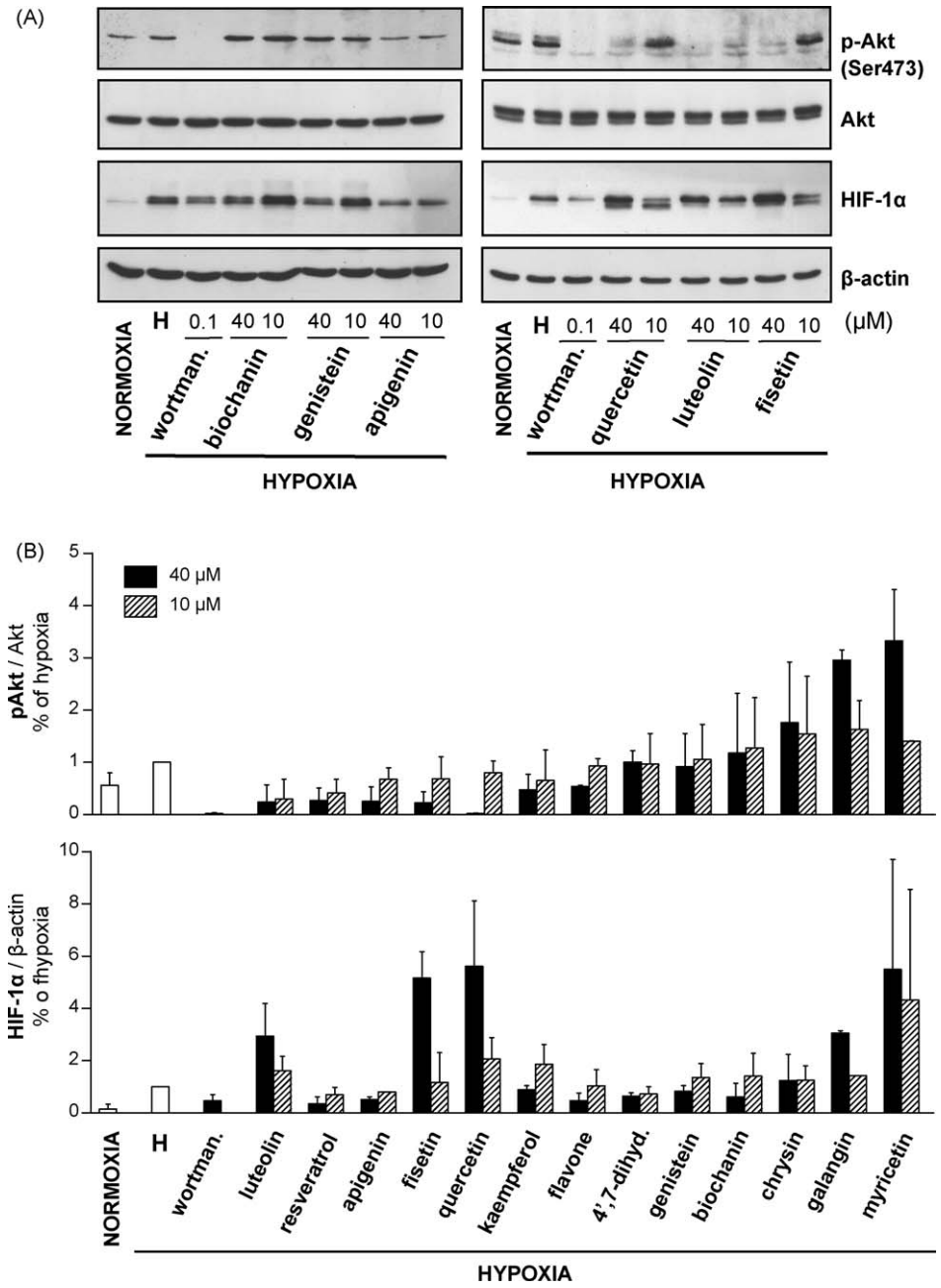


Fig. 2. Effect of flavonoids on Akt phosphorylation and HIF-1α expression in H157 cells cultured under hypoxia. (A) Subconfluent H157 cells were treated with either 40 or 10 μM of each flavonoid for 30 min followed by exposure to hypoxia for 6 h. Total cell extracts were analyzed by immunoblotting with antibodies against indicated proteins. (B) Mean ratio from densitometric analyses of pAkt(Ser^{473})/Akt and HIF-1α/β-actin from three independent experiments. The molecules are ordered according to their potency as inhibitors of Akt phosphorylation. Wortmannin, a PI3-kinase inhibitor, and resveratrol, a polyphenol that inhibits Akt phosphorylation and HIF-1α expression [5], were used as internal controls.

4. Discussion

We have analyzed the effect of 20 structurally related flavonoids on the induction of VEGF by hypoxia. As far as we know this is the first work in which their activity has been systematically compared. This is important not only to determine structure–activity relationships, that can lead to a better comparison of their potency and to the design of more active compounds, but also because the biological effects of flavonoids are pleiotropic. The comparative analysis of their mechanism of action can shed light on the relevant pathways by which these molecules affect VEGF expression in response to hypoxia.

Structure–activity relationships showed that flavones and flavonoles were the most effective VEGF inhibitors, while flavanones were inactive, showing the importance of the double bond in the C-ring for this activity (Tables 1 and 2). The presence of two hydroxyl groups in the A ring and the substitution of an additional hydroxyl group at the 4′-position of the B ring were important for this activity. For example, 5-hydroxyflavone or 7-hydroxyflavone were inactive, while chrysin (5,7-dihydroxyflavone) and 4′,7-dihydroxyflavone inhibited VEGF production with IC_{50} values of 11 μM and 15 μM, respectively. Adding a second hydroxyl group in the A ring at the 5-position decreased the IC_{50} to about 5 μM for apigenin (4′,5,7-trihydroxyflavone). Interestingly, the simultaneous presence of three hydroxyl groups at positions 3,

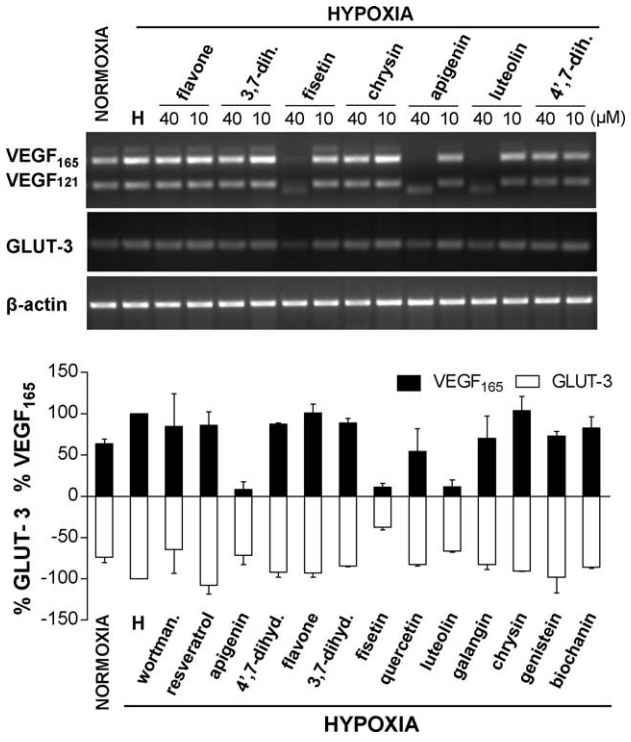


Fig. 3. Flavonoids inhibited VEGF transcription irrespective of total HIF-1α protein expression. H157 cells were pretreated with the indicated concentrations of flavonoids for 30 min followed by exposure to hypoxia for 24 h. VEGF and GLUT-3 mRNA levels were measured by PCR. Columns show the mean ratio of VEGF and GLUT-3 mRNA to β-actin from three independent experiments. Expression was normalized to those found in untreated cells under hypoxia.

5 and 7 seen in flavonols did not improve the inhibition of VEGF expression. Consistent with this observation, chrysin was more active than galangin (3,5,7-trihydroxyflavone), fisetin (3,3′,4′,7-tetrahydroxyflavone) was more efficient than quercetin (3,3′,4′,5,7-tetrahydroxyflavone), and apigenin surpassed kaempferol (3,4′,5,7-tetrahydroxyflavone) activity. It is well known that flavonoids also have pro-apoptotic properties, but the determinants for both activities are not the same. Specifically, the presence of a second hydroxyl substitution at the 3′-position in the B-ring did not reduced hypoxia-induced VEGF expression, but clearly increased cell cytotoxicity [32], as seen with luteolin, fisetin or quercetin with regard to apigenin (Table 2).

There are a large number of compounds that have been shown to inhibit the expression of HIF and VEGF. In fact, a variety of anticancer drugs, most of which were not developed as HIF-1 inhibitors, inhibit HIF-1 or VEGF expression. These include Hsp90 inhibitors, topoisomerase inhibitors, inhibitors of microtubule formation, histone deacetylase inhibitors, PI3-kinase inhibitors, soluble guanyl cyclase stimulators, thioredoxin inhibitors, and a number of other compounds with unexplained mechanisms of action [12]. Most of these compounds are not specific and, when analyzed in detail, they showed to exert their action by affecting a variety of cellular processes. For this reason we used resveratrol as a reference control. This is a well-known polyphenol which has been reported to share many mechanisms of action with flavonoids in other cellular processes, such as the induction of apoptosis. We also included wortmannin, a selective PI3-kinase inhibitor at nanomolar concentrations, since inhibition of the PI3-kinase pathway has been demonstrate to be central in the mechanism of action of many flavonoids.

It is commonly assumed that flavonoids inhibit hypoxia-induced VEGF expression by affecting HIF-1α protein levels. This hypothesis is mainly supported on studies with flavonoids that reduced the expression of both HIF-1α and VEGF, particularly apigenin [2–4,6,9]. Several works have demonstrated that apigenin may affect HIF-1α expression through impairing HIF-1α protein synthesis and reducing HIF-1α stability. For example, it was found that apigenin promotes HIF-1α degradation by affecting Hsp90 function and p53/Mdm2 pathway [2,3,9]. In addition, many active flavonoids inhibited the PI3-kinase/Akt pathway reducing HIF-1α protein synthesis through mTOR [3,4,7,9,33]. However, we have

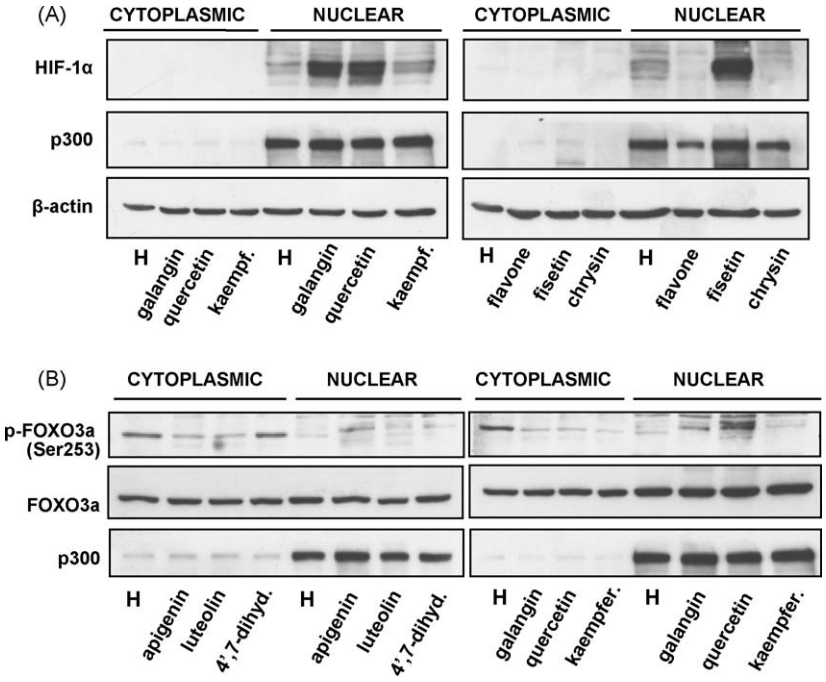


Fig. 4. Effect of flavonoids on nuclear localization of HIF-1α and FOXO3a. (A) H157 cells were pretreated with 40 μM of flavonoids for 30 min in serum-depleted medium and then exposed to hypoxia for 6 h. HIF-1α levels were detected by western blot as described above. β-Actin served as a loading control and p300 was used as a positive control for nuclear extracts. (B) Cells were treated as before and phosphorylated and total FOXO3a were determined by western blot in nuclear and cytoplasmic extracts.

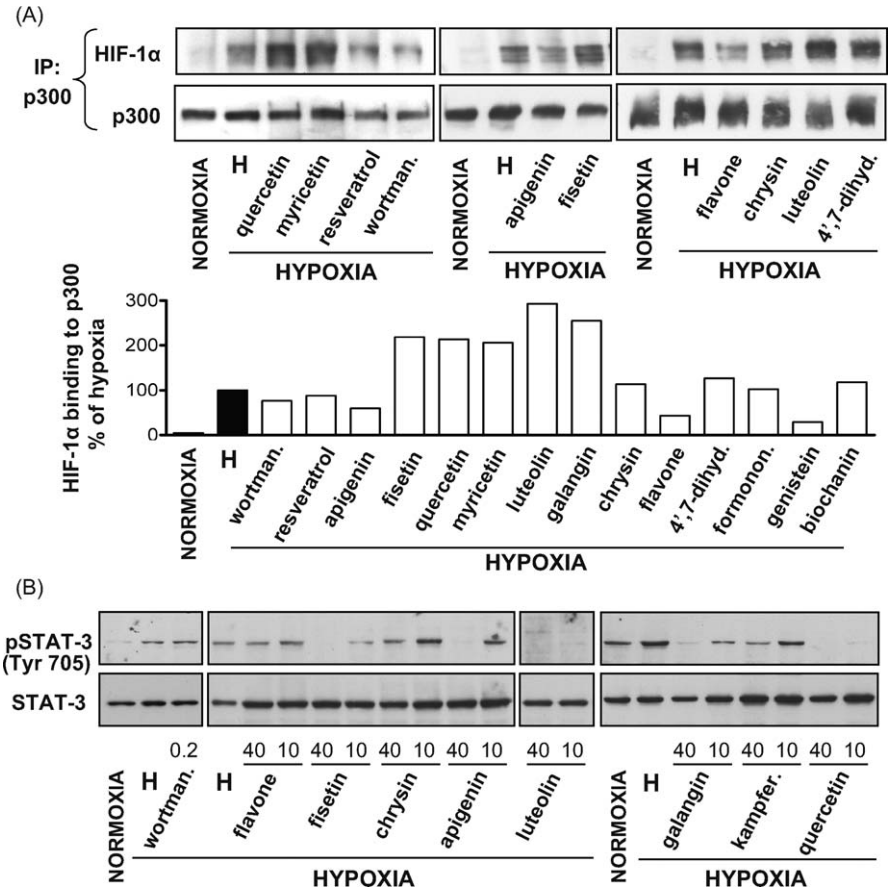


Fig. 5. Flavonoids did not block p300/HIF-1α binding in H157 cells but inhibited the activation of STAT3 by hypoxia. (A) To measure the amount of HIF-1α/p300 complex, H157 cells were treated with 20 μM of the indicated flavonoid for 30 min in serum-depleted medium and then exposed to hypoxia for 6 h. Cells were lysed and immunoprecipitated with anti-human p300 antibody followed by immunoblotting for HIF-1α. p300 was used as control for IP analyses. Columns show the ratio of HIF-1α to p300. (B) Effect of flavonoids on hypoxic activation of STAT3. H157 cells were pretreated with 40 or 10 μM of each flavonoid and then exposed to hypoxia for 6 h. Cell extracts were subjected to SDS-PAGE followed by immunoblotting with antibodies against tyrosine phosphorylated and total STAT3.

made a number of observations that indicate that flavonoids also affect hypoxia-induced VEGF expression by an alternative mechanism that did not depend on total HIF-1 protein levels. First, there is a poor correlation between the inhibition of HIF-1α and VEGF. For example, reference compounds that reduced HIF-1α expression as much as apigenin, such as wortmannin or resveratrol, are far less effective VEGF inhibitors than this flavonoid (Figs. 1 and 2). In addition, flavonoids reduced VEGF levels, but not HIF-1α levels, below those seen in normoxia (Fig. 1). More interestingly, compounds such as luteolin, fisetin or quercetin abolished hypoxia-induced VEGF protein expression at concentrations that induced HIF-1α.

Recently, it has been shown that some flavonoids, such as quercetin, fisetin and baicalein, stimulate HIF-1α expression under normal oxygen pressure. This induction is attributed to the metal-chelating properties of these flavonoids, that would deplete the intracellular iron ions needed by HIF-1/2α prolyl hydroxylases [28,34–38]. The induction of HIF-1α under hypoxia by flavonoids seen in this work is consistent with this observation, and indicate that prolyl hydroxylases have some function even at low oxygen tension [39]. However, there still remained the question of how compounds that increased HIF-1 expression under hypoxia inhibited VEGF expression. Interestingly, it has also been reported that the transcriptional activity of HIF-1 induced by flavonoids in normoxia was significantly lower than those observed with the iron-chelator deferoxamine [28]. This reduced transcriptional activity has been attributed to the inhibition by these flavonoids of the nuclear translocation of HIF-1α. Our results argue against this possibility in hypoxia. Isolation of cytoplasmic and nuclear fractions indicated that fisetin, quercetin or galangin increased nuclear HIF-1α in cells cultured under hypoxia. These observations are consistent with those previously found in normoxic conditions with quercetin, which stabilizes HIF-1α promoting its nuclear accumulation [34,37].

It was therefore possible that flavonoids would be affecting VEGF transcription by impairing the interaction between HIF-1 and p300. For example, it has been recently described that FOXO3a might be an important factor for hypoxia-induced gene expression, since it interferes with p300-dependent HIF-1 transcriptional activity [17]. Phosphorylation of FOXO factors by Akt prevents their transcriptional activity by promoting the export to the cytoplasm. Our data show that inhibition of Akt by flavonoids reduced FOXO3a phosphorylation in H157 cells, but total FOXO3a did not increase inside the nucleus. Perhaps the phosphorylation level was too low under the conditions employed and this regulation may be more important in other cell lines [29,30]. Although it was also reported that other modifications of HIF and or p300, such as hydroxylation, acetylation, nitrosylation and phosphorylation, may also affect the interaction between these factors, none of them seems to explain the inhibition of the HIF-1 transcriptional activity found with these flavonoids [2,12]. Direct measurement by immunoprecipitation of the amount of HIF bound to p300 clearly show that under hypoxic conditions flavonoids which induced HIF-1α also increased the concentration of this complex (Fig. 5).

At present it is not clear what mechanism is responsible for the inhibition of the transcriptional activity of HIF by these flavonoids. However, it is of interest that flavonoids that reduce VEGF expression inhibited the phosphorylation of STAT3 induced by hypoxia. STAT3 has been shown to play a critical role in promoting cancer cell survival, proliferation, tumor angiogenesis, metastasis, and tumor immune evasion/suppression [40]. STAT3 is activated by dimerization upon tyrosine phosphorylation in response to various cytokines and growth factors, and it is also a target of the c-Src, a non-receptor tyrosine kinase which is activated by hypoxia [16,41]. Inhibition of DNA binding by expression of either STAT3 or HIF-1α dominant negative mutants significantly reduces VEGF expression, indicating that both STAT3 and HIF-1α are needed for effective VEGF transcription [16]. Our results suggest that inhibition of STAT3 may be relevant for the inhibition by flavonoids of hypoxia-induced VEGF expression. Apigenin, which inhibited both HIF-1α and STAT3, are far more effective VEGF inhibitor than resveratrol or wortmannin, which only affected HIF-1α expression. On the other hand compounds that induced HIF-1α but abolished STAT3 phosphorylation, such as luteolin or fisetin, are potent VEGF inhibitors. Taken together results of this work support the notion that effective inhibition of VEGF expression induced by hypoxia would require the simultaneous inhibition of both HIF-1 dependent and HIF-1 independent pathways, and this should be taken into account in the search of more effective anti-VEGF compounds.

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